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PREFACE.

This thesis is an apologia. When I told my father in medicine, the late Professor W.T.Ritchie, that I had decided to join the Indian Medical Service he commented with dry humour, accompanied by a half smile and a characteristic lick of the lips, "you obviously want a life of hunting, shooting and fishing". It seems probable that this will be the last thesis to come from the pen of an officer of the Indian Medical Service, based on work carried out during his service career. Its presentation has been delayed by two major events in world history, namely the war and the transfer of power in India in 1947. The former interrupted the studies, and the latter caused a postponement of the writing due to the inevitable readjustment to life in a new scientific field.

My interest in malaria was somewhat casual during my early days in India, as I had formed the impression before leaving Edinburgh that quinine was the answer to the whole malaria problem. Hard experience soon dispelled this notion. My enthusiasm for the subject was first stimulated by a conversation in 1937 with a retired sub-assistant surgeon of the old school who had worked at Secunderabad for Sir Ronald Ross.

The studies presented in this thesis were carried out under the auspices of the Indian Research Fund Association, firstly before the war at the Pasteur Institute, Coonoor, and secondly after the war at the Central Research Institute, Kasauli. During both periods my director was Lt.Col.H.W.Mulligan,I.M.S. to whom I owe a great debt both for my training and for helpful guidance during the progress of the

experiments. The work throughout was on a collaboration basis being carried out by a team of workers, and it is neither possible nor desirable to assess the contribution that each made to the whole.

The greater part of the subject matter has already been published in scientific journals. This thesis is intended, however, to represent those aspects of our work which were of greatest interest to me. I am further conscious that in many places I must have drawn on ideas originally put forward by others and in some instances may have drawn on the work of others, but such would seem to be inevitable in team research. Consent to do so has been willingly given by all concerned. My principal colleagues in the work were Lt.Col. H.W.Mulligan, I.M.S., Capt.R.Passmore, I.M.S., and Dr.O.C.Lloyd. In conclusion it is a great pleasure to acknowledge the help of all the Indian personnel, too numerous to mention by name, with whom I served at Coonoor and Kasauli.

The life was good and abounded with professional opportunity: "I'd live it again".

INTRODUCTION.

The diversity of subjects listed in the table of contents to this thesis under the general title "Studies in Experimental Malaria" is more apparent than real. The central problems around which all the work has revolved and to which answers have been sought have been:-

What happens to the malarial sporozoite in the host? and How does the host react to malarial infection? It was but natural that subsidiary questions should arise during the course of such investigations.

The studies on the fate of the malarial sporozoite in the host formed a part of the work of the Mammalian Malaria Enquiry, which was located at Kasauli. This enquiry was formed in 1945 and was sponsored by the Government of India and the Royal Society. Its object was to attempt to determine whether or not exo-erythrocytic schizogony forms a part of the life cycle of the parasite in mammalian malaria. The scientific members of the enquiry were Lt.Col.H.W.Mulligan, I.M.S., Dr.O.C.Lloyd and myself. The infection studied was P.cynomolgi in M.mulatta. My responsibilities as part of the team of workers were the mosquito transmission of the infection to monkeys, and the experiments on the infectivity of the blood and tissues at different stages of the incubation period following sporozoite inoculation. In addition I was able to make observations on the course of the infection in the host.

The studies on the reaction of the host to malarial infection were conducted at Coonoor before the war. The fact that the Nutrition Research

Laboratories of the Indian Research Fund Association were located in the same building afforded an unique opportunity to investigate the effect of diet on the course of malarial infection. In this research I collaborated with Capt.R.Passmore, I.M.S.

The availability and cheapness of the monkey in India made the researches reported in this thesis possible. About 600 monkeys passed through my hands. We in India realised how fortunate we were in being able to study mammalian malaria under experimental conditions, unhampered by expense. The need for a malarial infection in a common laboratory mammal stimulated our interest in the infection which occurs naturally in the Malabar squirrel. The last essay of this thesis describes attempts to adapt P.ratufae to a common laboratory mammal.

For the sake of convenience in presentation this thesis has been arranged as a series of sections or chapters. In the opening section I have grouped together under the heading "Materials and Methods" all the technical procedures which were used throughout the work. The advantages in so doing are an avoidance of repetition and an aid to the presentation of the observations, results and conclusions in a series of straightforward essays, uninterrupted by technical details.

I have thought fit to record the earlier work as it was seen at the time it was done, and have made no attempt to compare it with work done subsequently in the same field.

SECTION I.

MATERIALS AND METHODS.

MATERIALS.

1. Plasmodia.

The strain of P.cynomolgi employed was originally isolated from M.irus by Sinton and Mulligan (1933) and described by Mulligan (1935). Since its isolation it has been passaged by blood inoculation frequently through M.mulatta monkeys, but passage by mosquito transmission has been carried out at relatively frequent intervals.

The strain of P.knowlesi employed was that described by Mulligan (1935). Since its isolation it has been passaged by blood inoculation only.

2. The Vertebrate Hosts.

Two common species of Indian monkeys were used namely M.mulatta and M.radiata. Although the age of the monkeys was not known definitely, it may be stated in general terms that they were mostly young adolescents. The former species was obtained from dealers in the vicinity of Ambala in the Punjab and of the many hundreds examined from this area no natural infection has been detected. The latter species was obtained from a dealer who caught the monkeys in the foot-hills of the Nilgiri Hills in South India.

3. The Mosquito Hosts.

As no"colonised" anopheline vector was available in India at the time all mosquitoes used were bred from larvae taken from breeding places in the field. A.annularis and A.subpictus were the chief vectors employed in the experimental mosquito transmission of P.cynomolgi. This choice was based on the high prevalence in the plains of Northern India of

the former species during the spring and of the latter species during the autumn.

METHODS.

1. Mosquito Transmission.

(a). Supply of normal Mosquitoes.

The normal mosquitoes were reared from larvae which were obtained from breeding places in the field. The supply of larvae was maintained by collections twice daily. They were transported from the breeding sites to the laboratory in large buckets. After arrival at the laboratory they were transferred from the buckets to large enamel basins, care being taken to remove the predators and to avoid overcrowding in the larval colony. During the period of development of the adult mosquitoes from larvae the enamel basins containing the larval colony were kept inside a mosquito cage 3' x 3' x 3' in size. The larval diet consisted of algae which were included in the collection from the breeding place. No supplements to this diet were provided. This diet proved to be satisfactory as judged by the low mortality in the colony. Newly hatched adults were collected daily and transferred to a separate mosquito cage. They were kept for 48 hours before they were allowed to feed on a suitable gametocyte carrier. No food was provided during this period but water was supplied. It was found by experience that newly hatched adults did not feed as well on the gametocyte carrier as those which were maintained for 48 hours after pupation. This interval between pupation and the blood feed enabled the mouth parts to harden. The mortality rate at this stage varied with the prevailing climatic conditions.

(b). Technique of Infecting Mosquitoes.

(i). Provision of gametocyte carriers.

The gametocyte carriers were M.mulatta monkeys infected with P.cynomolgi. An adequate supply was ensured by calculating anticipated demands on the basis of the numbers of normal mosquitoes available and infecting the required number of monkeys by blood subinoculation. During a transmission season of 8 weeks when batches of normal mosquitoes were fed nearly every day a new monkey had to be infected every 3 - 4 days. Although the majority of the gametocyte carriers which were used were infected by blood subinoculation, in a few cases monkeys with a sporozoite induced infection were employed. In the early stages of the work the selection of a suitable gametocyte carrier was based on the criterion of the presence of 10 or more gametocytes per 10,000 red cells, but in the later stages of the work daily feedings were carried out on the same gametocyte carrier from the beginning of parasitaemia throughout the course of the primary attack. This latter plan was adopted due to the recognition early in the work of our inability to recognise a good "infector" for mosquitoes.

(ii) Method of Infecting Normal Mosquitoes.

A trussed gametocyte carrier, with a shaved abdomen and thorax, was introduced into a large mosquito cage containing 48 hours old starved normal mosquitoes. These were allowed to feed for ½ hour in the dark. At the end of this period the monkey was removed and the mosquitoes which had taken a blood meal were identified, collected, counted and transferred to Barraud's cages in the proportion of 50 per cage.

(c). Conservation of Infected Mosquitoes.

During the period of the sporogony cycle of the parasite the mosquitoes were stored in a dark room in which the temperature was not controlled but in which the relative humidity was maintained at about 80 per cent. This was done by keeping moist sand on the floor. Maximum and minimum temperature readings were recorded twice daily at 8.a.m. and 4.p.m. The Burraud's cages containing the mosquitoes were stored on racks specially made for the purpose and beneath each cage was placed a large pad of cotton wool which was kept soaked with water. The mosquitoes' diet consisted of raisins and water, and no further blood meal was given. A small handful of raisins was placed on the top of each cage along with a pad of lint soaked in water. In the early stages of the work a 10% solution of glucose was tried as an article of diet but this proved to be unsatisfactory, due to the difficulty experienced in starving the batch prior to use in the transmission of the infection to the vertebrate host. This difficulty was accounted for by the fact that the glucose solution had impregnated the cloth of the cage. Those batches which had been provided with a diet of glucose in solution showed little avidity for a blood meal compared with the batches which had been fed on raisins.

Dead mosquitoes were removed from the cages daily, counted and recorded.

(d). Sample Dissections.

The index of experimental infection of each batch and the progress of the sporogony cycle were controlled by sample dissections. This method of sampling consisted in the dissection of the mid-gut and glands of about 5% of the mosquito batch.

(e). Methods of Transmission to the Vertebrate Host.

(1). By mosquito bite.

An interval of 2 - 3 days was allowed to elapse after the first appearance of sporozoites in the salivary glands before the mosquito batch was used to transmit the infection to monkeys. This was done to ensure that the salivary glands of the majority of the mosquitoes in the batch had a heavy sporozoite infection at the time of the infecting feed. Prior to the feed each batch was starved for 24 hours. The technique which was followed with batches of 100 or less was to hold the Burraud's cage tightly across the shaved abdomen of the monkey which it was desired to infect. The mosquitoes were allowed to feed for ½ hour in the dark. At the end of this period the cage was removed from the monkey's abdomen, and the mosquitoes which had not fed were identified, removed from the cage and counted. When more than 100 mosquitoes were used in transmitting the infection to the vertebrate host the "truss" method of introducing the monkey into a large mosquito cage which contained the infected mosquitoes was employed.

(ii). By Inoculation of Sporozoites.

The choice of the method employed in the preparation of a suspension of sporozoites from infected mosquitoes was based largely on the availability of infected mosquitoes. When large numbers were available a suspension of ground-up mosquito thoraces and abdomens was used. This had the advantage that it could be easily and quickly prepared. When small numbers were available due to a high mortality among the mosquito population, such as occurred during the autumn of 1945, the sporozoite suspension was prepared by dissection of the salivary glands of the mosquito.

The reliability of the techniques which were followed and are described below, was proved by successful transmission to monkeys on numerous occasions.

The method which was adopted in the preparation of a sporozoite suspension with the thoraces and abdomens of infected mosquitoes consisted in grinding them in a serum-saline mixture. The technique was carried out in several stages beginning with the excision of the wings and legs of mosquitoes which were stunned by percussion prior to the operation. This stage was completed for all specimens before passing on to the excision of the head of each mosquito in the batch with dissecting needles. Care was taken during the excision of the head to avoid extracting the salivary glands from the thorax. As each thorax and abdomen was isolated it was placed in a small agate mortar containing 0.2 ml. of equal parts of normal monkey serum and physiological saline. The final stage involved grinding the thoraces and abdomens with a small agate pestle until an even suspension was formed. Grinding was carried out for about 5 minutes. The final suspension was a dark brown-black fluid. When the suspension was too thick and viscid more serum-saline mixture was added to enable it to be sucked up into the tuberculin syringe which was fitted with a No.27 gauge needle. The time which elapsed from the commencement of the preparation of the suspension to the end of the injection was about ½ hour with a batch of 50 mosquitoes and when two technicians were employed to isolate the thoraces.

The method of preparation of a sporozoite inoculum using the salivary glands of infected mosquitoes was to dissect the glands on a slide in a mixture of equal parts of normal monkey serum and physiological saline. On completing the dissection the glands were sucked up into a No.27 gauge needle along with the fluid in which they had been dissected. The needle was attached to a tuberculin syringe containing 0.1 ml. of serum-saline mixture. As far as possible the glands were kept in the bore of the needle and were not allowed to enter the barrel of the syringe. The injection was usually completed within 10 minutes from the commencement of dissection and in no case was it longer than ½ hour.

2. Technique of Cardiac Puncture.

Cardiac puncture proved the most satisfactory method of obtaining large quantities of blood. It was the method employed in the studies on the infectivity of the blood at different stages of the incubation period following sporozoite inoculation of P.cynomolgi and for the collection of quantities of immune sera. A 20 c.c. syringe containing 2 - 3 ccs. citrate saline was used. The donor monkey is anaesthetised with chloroform and the needle of the syringe introduced through the right intercostal space close to the sternum. After insertion through the skin the needle is pushed gently inwards, downwards and to the left until the pulsation of the heart on the point of the needle is felt. It is then pushed sharply into the heart and blood withdrawn into the barrel of the syringe.

3. Technique of Splenectomy.

Splenectomy in the monkey proved to be a simple procedure. The animal is anaesthetised with

chloroform and the approach to the spleen made through a left upper paramedian incision. On opening the peritoneum the spleen is located and drawn to the surface of the wound. The pedicle is clamped, ligated with two separate catgut sutures and divided between the sutures. The clamp is then released and any bleeding points dealt with before returning the pedicle to the peritoneal cavity. The peritoneum is then closed and the abdominal wall closed in layers with a continuous suture for each layer. No special post operative care is required.

In one series of experiments the spleen was transplanted into the abdominal wall and portions of its substance excised daily for examination. Haemorrhage was controlled by cauterisation.

4. Method of Tissue Subinoculation.

The subinoculation of splenic tissue was carried out either by transplantation to the peritoneal cavity of the recipient monkey or by the intraperitoneal injection of a suspension prepared by grinding the spleen in a mortar and suspending it in normal saline solution. Spleen transplants were introduced into the peritoneal cavity of the recipient monkeys through a left lower paramedian incision. Before closing the peritoneal cavity the transplant was wrapped in the greater omentum. The transplantation was completed within 15 - 20 minutes from the removal of the spleen from the donor.

In the subinoculation experiments with tissues other than spleen the donor monkey was sacrificed and portions of the organs required for investigation removed under aseptic conditions. A saline suspension was prepared by grinding the

portion of excised organ in a mortar. This suspension was injected intraperitoneally into the recipient monkey. In all cases the subinoculation was completed within 1 - 2 hours of removal from the donor.

In the subinoculation experiments with blood the injection of the recipient monkey was completed within 10 - 15 minutes of removal of the blood from the donor.

5. Blood Film Routine.

During the incubation period the thick smear method was used as a routine for the detection of parasites in the peripheral blood. In the cases in which infection did not develop after sporozoite inoculation thick smears were examined daily for 21 days, thereafter examinations three times a week were carried out

During the period of the acute attack and during relapses, thin films were taken daily at 8.a.m. 12 noon, 4 p.m., and 8.p.m. The number of parasites in each film was estimated against 10,000 red blood cells, and the average of the four daily parasite counts was taken as the index of parasite prevalence for that day.

During the chronic phase of the infection thick smears were examined three times a week as a routine. The stains employed for blood films were either Giemsa or J.S.B. as recommended by Jaswant Singh and Bhattacharji (1944). The latter had the advantages that the reagents were easily obtained during the war and it is a speedier method, a matter of no little importance when large numbers of blood films are required to be stained each day. J.S.B. stain proved a satisfactory method of staining.

SECTION II.

OBSERVATIONS ON THE INFECTIVITY OF TISSUES OF MACACA MULATTA DURING THE INCUBATION PERIOD FOLLOWING EXPOSURE TO INFECTION WITH SPOROZOITES OF PLASMODIUM CYNOMOLGI.

INTRODUCTION.

The fate of the malarial sporozoite during the incubation period of the disease in the vertebrate host has long been a puzzle to parasitologists. Schaudinn's (1902) observation that the sporozoite shortly after its inoculation by the infected mosquito enters the red cell has never been confirmed. James and Tate (1938) opened up a new field of enquiry when they reported the occurrence of exo-erythrocytic schizogony in the reticulo-endothelial cells of chicks infected with P.gallinaceum. The development of these exo-erythrocytic schizonts from sporozoites of P.gallinaceum was clearly demonstrated by Huff and Coulston (1944). It remained, however, a vexed question as to whether or not these observations on avian malaria could be applied to mammalian malaria. Strong arguments in favour of a pre-erythrocytic cycle of parasite development in mammalian malaria were advanced on the basis of chemotherapeutic experience, and on infectivity studies of the blood during the incubation period following exposure to infection with sporozoites (Fairley 1945; Davey 1946). Attempts to demonstrate such pre-erythrocytic forms were either unsuccessful (Huff and Coulston 1947) or unconvincing (Raffaele 1937,1940; Brug 1940).

Since the work which will be presented in this section was carried out, Shortt and his colleagues at the London School of Hygiene and Tropical Medicine have clearly demonstrated the occurrence of pre-erythrocytic schizonts in the liver in infections with

P.cynomolgi and P.vivax. (Shortt et al 1948a: 1948b: Shortt and Garnham 1948c; 1948d).

The experiments which form the subject matter of this section were carried out at the same time as a large scale unsuccessful histological investigation designed to determine whether or not pre-erythrocytic forms of P.cynomolgi could be demonstrated in M.mulatta. Their chief aim was to try and obtain a lead on the important questions:- What tissue would best repay a detailed search? and what stage of the incubation period after sporozoite inoculation are pre-erythrocytic forms present in the greatest number? It was considered that by this means the histological side of the enquiry might be helped. With this end in view, and on the assumption that such forms, if present, would be infective on subinoculation to susceptible animals, as had shown to be the case in avian malaria (Coulston et al 1945), the series of experiments to be described below were undertaken.

RESULTS.

For the sake of convenience in reading this thesis the tables have been grouped at the end of the section.

1. Subinoculation of blood taken from monkeys within 16 hours of infection with sporozoites.

Blood from monkeys infected with sporozoites in various ways was withdrawn at spaced intervals after the injection of sporozoites and inoculated into clean monkeys by various routes. Details of these experiments and of the results observed are summarised in Table I.

Donor monkeys were infected by mosquito bite (3 experiments) or by the injection of suspensions of sporozoites administered by the subcutaneous (14 experiments) or intradermal (3 experiments) routes. The

number of mosquitoes used to infect the donors varied from 8 to 114 (average 48). All the donor monkeys, except two which died during the incubation period, ultimately developed patent malarial infections. The two which died have been recorded as positive since other monkeys bitten at the same time by mosquitoes from the same batch developed malaria.

The time interval elapsing between sporozoite injection and withdrawal of blood for subinoculation varied from less than 1 minute up to 16 hours.

Eighteen of the subinoculated monkeys failed to develop malaria during an observation period varying from 34 to 98 days, and despite the fact that eight of them were splenectomised as a test for latent infection. Two of the subinoculated monkeys developed patent infections, one of these (E-392) received 20 ml. of blood intravenously from a donor (E-391) 20 minutes after the subcutaneous injection of a suspension of the thoraces and guts of 114 infected mosquitoes., The other (E-385) received 15 ml. of blood intravenously from a donor (E-386) 45 minutes after the subcutaneous injection of a suspension of thoraces and guts from 100 infected mosquitoes.

These experiments provide clear evidence of the infectivity of the blood up to 45 minutes following the injection of large numbers of sporozoites by the subcutaneous route, and may be presumed to be due to the presence of viable sporozoites in the blood stream.

2. <u>Subinoculation of blood taken from monkeys at daily intervals after the injection of sporozoites</u>.

These experiments were an extension of those described above and summarised in Table I. Blood was taken from monkeys at daily intervals (two or more observations for each day up to 10 days) after the

after the injection of sporozoites. Details of the experiments and of the results obtained are summarised in Table II.

In all, 35 subinoculation experiments were made. The donors received sporozoites by mosquito-bite, (19 experiments) or by the injection of sporozoite suspensions by the subcutaneous (11 experiments), intravenous (3 experiments) and intr-splenic (2 experiments) routes. The number of infected mosquitoes used varied widely but was in excess of 50 in 17 of the experiments. All of the 31 donors which survived long enough developed patent malarial infections, of the remaining 4, three were sacrificed and one died during the incubation period. These four animals have been recorded as positive since other monkeys infected from the same batch of mosquitoes at the same time developed patent malaria.

Quantities of blood varying from 7 ml. to 20 ml. were subinoculated into clean monkeys by the intraperitoneal (26 experiments), intravenous (8 experiments), and the intramuscular (1 experiment) routes.

In no case was the blood of monkeys taken at intervals varying from 1 to 8 days found to be infective when subinoculated to clean monkeys. The latter were observed for periods ranging from 31 to 112 days; in one case (E-459) which received blood from a donor injected with sporozoites 8 days previously no evidence of malaria was observed after splenectomy.

In every one of the 10 experiments in which blood for subinoculation was taken after a lapse of more than 8 (9 to 14) days following the injection of sporozoites, the recipients developed

patent malarial infections.

Conclusions from blood subinoculation experiments.

From the experiments summarised in Tables I and II, it seems reasonable to conclude that, in M.mulatta infected with sporozoites of P.cynomolgi, viable sporozoites may be present in the blood stream for a relatively short time after the subcutaneous injection of large numbers of sporozoites, the maximum observed period being 45 minutes. During the next 8 days, that is, during the remainder of the incubation period, the blood is consistently non-infective when subinoculated into clean monkeys. From the 9th day onwards the blood is consistently infective when subinoculated to clean monkeys. In some cases the blood may be infective on subinoculation from 1 to 3 days before erythrocytic forms of the parasite are detectable by routine thick film examination.

3. Subinoculation of spleen tissue from monkeys previously injected with sporozoites.

In these experiments spleen tissue was removed from monkeys at intervals varying from 2 to 14 days following the injection of sporozoites and subinoculated into clean monkeys. Details of the experiments and the results observed are given in Table III.

The twelve donor monkeys were infected by mosquito bite (7 experiments) or by the injection of suspensions of sporozoites given intravenously (3 experiments) or implanted directly into the spleen (2 experiments). All but three of these monkeys developed patent malarial infections, the three exceptions being animals sacrificed during the incubation period. These have been recorded as positive in the

protocols since other monkeys bitten by the same batch of mosquitoes at the same time contracted the infection.

Spleen tissue varying in amount from the whole spleen to a fraction of approximately 1/6 of the spleen was implanted intraperitoneally either by direct transplant at open operation or by the injection of a suspension of spleen tissue prepared by the method already described.

Immediately prior to removal of spleen tissue from the donor by biopsy or splenectomy, blood from the donor was inoculated into a clean monkey as a control of the infectivity of the blood ("blood control").

None of the 9 monkeys which were subinoculate with spleen tissue removed from the donors at intervals varying from 2 to 10 days following the introduction of sporozoites developed malaria within observation periods varying from 30 to 63 days. On the other hand all of the three monkeys which were subinoculated with spleen tissue removed from monkeys between 12 and 14 days following the introduction of sporozoites developed patent infections.

Subinoculation of a small fraction (1/6) of the spleen of one monkey (E-147) failed to produce infection in the recipient (E-99) when blood removed at the same time produced infection in another monkey. The probable explanation of this apparent discrepancy is that the dosage of blood administered was many times greater than the dosage of spleen tissue.

An observation of interest was that transplantation of the whole spleen from monkeys E-282 and E-264 at intervals of 2 and 4 days respectively following intrasplenic injection of large numbers of

sporozoites failed to produce infection in the subinoculated animals.

Conclusion from spleen subinoculation experiments.

From the experiments summarised in Table III it may reasonably be concluded that, in M.mulatta infected with sporozoites of P.cynomolgi, spleen tissue is non-infective to subinoculated animals after the lapse of 2 days from the time of exposure to infection and thereafter throughout the whole of the incubation period.

4. Subinoculation of other tissues taken from monkeys following exposure to infection with sporozoites.

In these experiments suspensions of various tissues taken from monkeys at intervals during the incubation period following the injection of sporozoites were subinoculated to clean monkeys. The tissues subinoculated included blood, liver, bone marrow, brain, lung, kidney, suprarenal, pituitary and striped muscle. Details of these experiments and of the results observed are summarised in Table IV.

Three donor monkeys (E-310, E-256 and E-116) were infected by the bites of 65, 60 and 5 infected mosquitoes, respectively. Monkey E-310 was sacrificed on the 5th day, E-256 on the 7th day and E-116 on the 8th day following exposure to infection and their tissues, removed under aseptic conditions andsuspended in physiological saline solution, were injected intraperitoneally into clean monkeys, a separate animal being used for the subinoculation of each tissue. None of the subinoculated monkeys developed malaria within observation periods varying from 35 to 116 days. As the three donor monkeys were sacrificed during the incubation period, there is no absolute proof that

they had been successfully infected, but they have been presumed to be positive and recorded as such in the protocols, since other animals bitten at the same time by mosquitoes of the same batch developed malaria.

Similar experiments were carried out with monkeys E-133 (blood, liver, bone marrow, brain, lung, kidney, muscle) on 13th day, and monkey E-134 (blood, brain) on 14th day following exposure to infection. All of the tissues from these two donors produced malaria in the subinoculated monkeys, except the bone marrow of E-133.

Conclusion from tissue subinoculation experiments.

The results of these experiments indicate that in M.mulatta exposed to infection with sporozoites of P.cynomolgi neither the blood nor any other tissues subinoculated to clean monkeys is infective at the stages at which observations were made, namely the 5th, 7th, and 8th days of the incubation period. Once the blood becomes infective, other tissues are also infective, presumably by reason of the parasitized erythrocytes contained in them.

DISCUSSION.

After months of patient search for pre-erythrocytic forms of P.cynomolgi the histologist on the Mammalian Malaria Enquiry impatiently stated that it was like looking for a needle in a haystack - some needle and what a haystack!! It was a problem not only in space but of time. What tissue would best repay study and at what stage of the incubation period? The experiments described above failed in their object, namely, to give a lead to the histologist, thereby enabling him to carry out an intensive search of a particular organ rather

than an extensive reconnaisance of many organs. They are, however, of interest in relation to the results obtained from similar work in avian malaria, and more especially with regard to the demonstration of pre-erythrocytic forms of P.cynomolgi in monkey liver following the injection of massive doses of sporozoites (Shortt et al, loc.cit).

Re-examination of our histological material taken from monkeys at all stages of the incubation period following injections of large doses of sporozoites has failed to reveal the presence of pre-erythrocytic schizogony in the liver or elsewhere. There is, therefore, no absolute proof that the tissues used for the subinoculation experiments described above actually contained pre-erythrocytic forms. Failure to detect these forms at microscopical examination does not, of course, mean that there were none present; it is a common experience to find that blood is infective on subinoculation when the presence of parasites cannot be detected by routine thick-film examinations. When it is remembered that the doses of sporozoites given was very considerable (often more than 50 infected mosquitoes) and the quantities of various tissues used for subinoculation was relatively enormous, it seems reasonable to assume that pre-erythrocytic forms were, in fact, present in at least some of the tissues used in these experiments. Up to the present, pre-erythrocytic forms of P.cynomolgi have been observed only in the liver but there is as yet no proof that they do occur elsewhere albeit in insufficient numbers to be readily detected at histological examination.

The consistent failure to produce infection in subinoculated monkeys by the injection of tissues from animals within 8 days of exposure to infection with sporozoites (except the blood up to 45 minutes) appears

to warrant the conclusion that pre-erythrocytic forms of P.cynomolgi are non-infective to animals susceptible to infection with either sprozoites or trophozoites. This conclusion receives the strongest support from the observation of Shortt et al (1948a) that liver tissue known to contain pre-erythrocytic forms of P.cynomolgi failed to produce infection in a sub-inoculated animal.

The finding that tissues are consistently non-infective on subinoculation during the incubation period in mammalian malaria is of special interest in relation to previous findings in avian malaria. Numerous authors have reported that, in avian malaria, various tissues are infective during the incubation period following sporozoite injection and at a time when the blood is non-infective (Warren and Coggeshall 1937; Kikuth and Mudrow, 1938; de Court and Schneider, 1938: Coulston et al, 1945). Tissues which have been shown to be infective during this phase include spleen, liver, kidney, brain, ovary, lymph nodes and thymus. Coulston et al (loc.cit), in studies on P.gallinaceum, demonstrated that, in infections produced by the bites of infected mosquitoes, the only tissues which were infective during the 36 hours following exposure to infection were the skin at the site of biting and the spleen, whereas following intravenous injection of sporozoites many other tissues including heart, brain, thymus, intestine and bone marrow, were infective during the same period. These findings in avian malaria are in marked contrast with what is at present known of mammalian malaria and require elucidation.

As regards the infectivity of the blood following exposure to infection with sporozoites there is a close parallel between avian, simian and human

malaria. In infections with P.gallinaceum, Coulston et al (loc. cit.) found that the blood was infective during the first 20 minutes following sporozoite injection and again 36 hours later. This latter period of infectivity coincided with the liberation of merozoites from cells of the lymphoid-macrophage systems. In our experiments with P.cynomolgi the blood was prove to be infective on subinoculation at intervals of 20 minutes and 45 minutes following subcutaneous injection of large numbers of sporozoites but was subsequently negative during the remainder of the incubation period. It is reasonable to suppose that the infectivity of the blood at this early stage is due to the presence of viable sporozoites. These findings are very similar to those reported by Fairley (1945) who observed that in human volunteers infected with P.vivax and P.falciparum the blood was infective for about half an hour after injection of sporozoites and then remained negative throughout the rest of the incubation period.

The duration of the incubation period as estimated by the infectivity of the blood on subinoculation was found by Fairley (loc. cit.) to be 6 days for P.falciparum and 8 days for P.vivax. The duration of the incubation period of P.cynomolgi in M.mulatta has been found to be 9 days as estimated by the subinoculation experiments described above. This coincides exactly with the direct observations on pre-erythrocytic schizogony of this species of Plasmodium reported by Shortt and Garnham.

TABLE I.

PROTOCOLS OF EXPERIMENTS IN WHICH BLOOD WAS SUBINOCULATED WITHIN 16 HOURS OF SPOROZOITE INOCULATION.

Recipient Monkey Number	Donor Monkey Number	Time interval between sporozoite injection and withdrawal of blood for subinoculation	Method of administration of sporozoites to donor. *	No. of infected mosquitoes used to infect donor £	Amount of blood in millilitres subinoculated intravenously (iv) intramuscularly (im) or intraperitoneally (ip).	Results. (+ malaria: - no malaria.) Donor	Recipient	%
E-415	E-414	Immediate	Subcutaneous (Thoraces & gut)	26	20 (iv)	+	-	(86) Spl.
E-427	E-426	Immediate	-do-	29	20 (iv)	+	-	(85) Spl.
E-440	E-439	1½ minutes	-do-	39	12 (iv)	+	-	(81) Spl.
E-418	E-417	2 minutes	-do-	60	20 (im)	Died ‡	-	(86) Spl.
E-384	E-386	5 minutes	-do-	100	13 (im)	+	-	(98) Spl.
E-434	E-433	5 minutes	-do-	30	20 (iv)	+	-	(81) Spl.
E-160	E-159	5 minutes	Mosquito bite	11	10 (ip)	+	-	(36)
E-436	E-435	8 minutes	Subcutaneous (Thoraces & gut)	25	20 (iv)	+	-	(81) Spl.
E-171	E-170	10 minutes	Intradermal (Salivary Glands)	8	9.5 (ip)	+	-	(35)
E-281	E-270	10 minutes	Intradermal (Thoraces)	80	20 (ip)	+	-	(63)
E-392	E-391	20 minutes	Subcutaneous (Thoraces & gut)	114	20 (iv)	+	+	
E-161	E-159	25 minutes	Mosquito bite	11	6 (ip)	+	-	(36)
E-393	E-391	30 minutes	Subcutaneous (Thoraces & gut)	114	3 (iv)	+	-	(44)
E-451	E-450	30 minutes	-do-	58	20 (iv)	Died ‡	-	(80) Spl.
E-172	E-170	37 minutes	Intradermal (Salivary Glands)	8	10.5 (ip)	+	-	(35)
E-385	E-386	45 minutes	Subcutaneous (Thoraces & gut)	100	15 (iv)	+	±	
E-163	E-159	72 minutes	Mosquito bite	11	12 (ip)	+	-	(36)
E-442	E-435	4 hours	Subcutaneous (Thoraces & gut)	25	20 (iv)	+	-	(35)
E-446	E-433	8 hours	-do-	30	20 (iv)	+	-	(35)
E-448	E-439	16 hours	-do-	78	15 (iv)	+	-	(34)

* The mosquito tissue in parenthesis indicates the source from which the sporozoites were obtained, thus, (thoraces and gut) signifies the entire contents of the thorax including salivary glands together with the midgut.

£ The number of infected mosquitoes stated was calculated by multiplying the number actually used by the index of infection for the batch. In all cases, therefore, the number actually used was equal to, or greater than, the numbers given in the protocols.

‡ Donor died during incubation period but control bitten by mosquitoes from same batch developed malaria.

% The number in parenthesis indicates the period in days during which the recipient monkey was observed.

Spl. Indicates that malaria did not develop after splenectomy.

TABLE II.

PROTOCOLS OF EXPERIMENTS IN WHICH BLOOD WAS SUBINOCULATED AT DAILY INTERVALS FOLLOWING SPOROZOITE INJECTION.

Recipient Monkey Number.	Donor Monkey Number.	Time interval in days between sporozoite injection and removal of tissue for subinoculation	Method of administration of sporozoites to donor monkey. *	No. of infected mosquitoes used to infect donor monkey £	Amount of blood in millilitres subinoculated intravenously (iv), intramuscularly (im) or intraperitoneally (ip).	Results (+ malaria: - no malaria) Donor	Recipient %
E-303	E-300	1	Mosquito Bite	36	15 (ip)	+	- (56)
E-387	E-386	1	Subcutaneous (Thoraces & gut)	100	20 (im)	+	- (49)
E-431	E-426	1	-do-	118	10 (iv)	+	- (36)
E-307	E-301	2	Mosquito Bite	35	15 (ip)	+	- (55)
E-293	E-282	2	Intrasplenic (Thoraces)	79	20 (ip)	+	- (61)
E-456	E-450	2	Subcutaneous (Thoraces & gut)	58	20 (iv)	Died ‡	- (31)
E-309	E-300	3	Mosquito Bite	36	15 (ip)	+	- (54)
E-432	E-426	3	Subcutaneous (Thoraces & gut)	118	20 (iv)	+	- (35)
E-323	E-301	4	Mosquito Bite	35	20 (ip)	+	- (53)
E-295	E-264	4	Intrasplenic (Thoraces)	64	15 (ip)	+	- (61)
E-438	E-426	4	Subcutaneous (Thoraces & gut)	118	20 (iv)	+	- (34)
E-324	E-300	5	Mosquito Bite	36	20 (ip)	+	- (52)
E-330	E-310	5	Mosquito Bite	65	20 (ip)	+ ‡	- (48)
E-449	E-426	5	Subcutaneous (Thoraces & Gut)	118	20 (iv)	+	- (33)
E-326	E-301	6	Mosquito Bite	35	20 (ip)	+	- (51)
E-104	E-95	6	Intravenous (Salivary Glands)	1	7 (ip)	+	- (112)
E-268	E-257	6	Mosquito Bite	100	20 (ip)	+	- (63)
E-454	E-426	6	Subcutaneous (Thoraces & gut)	118	20 (iv)	+	- (32)
E-327	E-300	7	Mosquito Bite	36	15 (ip)	+	- (50)
E-267	E-253	7	Mosquito Bite	79	20 (ip)	+	- (63)
E-283	E-256	7	Mosquito Bite	60	17 (ip)	+ ‡	- (62)
E-455	E-426	7	Subcutaneous (Thoraces & gut)	118	20 (iv)	+	- (78)
E-329	E-301	8	Mosquito Bite	35	20 (ip)	+	- (49)
E-137	E-116	8	Mosquito Bite	5	15 (ip)	+ ‡	- (32)
E-459	E-426	8	Subcutaneous (Thoraces & gut)	118	20 (ip)	+	- (77) Spl.
E-338	E-300	9	Mosquito Bite	36	15 (ip)	+	+
E-460	E-426 8	9	Subcutaneous (Thoraces & gut)	118	20 (ip)	+	+
E-340	E-301	10	Mosquito Bite	35	18 (ip)	+	+
E-81	E-147	10	Mosquito Bite	4	12 (ip)	+	+

.....cont'd.

TABLE II.

Continued.....

Recipient Monkey Number,	Donor Monkey Number	Time interval in days between sporozoite injection and removal of tissue for subinoculation	Method of administration of sporozoites to donor monkey. *	No. of infected mosquitoes used to infect donor monkey £	Amount of blood in millilitres subinoculated intravenously (iv), intramuscularly (im) or intraperitoneally (ip).	Results (+ malaria: - no malaria) Donor	Recipient %
E-461	E-426	10	Subcutaneous (Thoraces & gut)	118	17 (iv)	+	+
E-341	E-300	11	Mosquito Bite	36	18 (ip)	+	+
E-342	E-301	12	Mosquito Bite	35	18 (ip)	+	+
E-94	E-144	12	Intravenous (Salivary Glands)	4	10 (ip)	+	+
E-152	E-133	13	- do -	2	12 (ip)	+	+
E-167	E-134	14	Mosquito Bite	13	12 (ip)	+	+

* The mosquito tissue in parenthesis indicates the source from which sporozoites were obtained, thus: (Salivary Glands) signifies dissected glands, (Thoraces) the entire thoracic contents including glands, and (gut) the dissected mid-gut.

£ The number of <u>infected</u> mosquitoes was calculated by multiplying the actual number used by the index of experimental infection for the batch.

‡ These monkeys were sacrificed or died of other causes during the incubation period but have been recorded as positive since other monkeys on which the same batch of mosquitoes fed at the same time developed malaria.

% The figure in parenthesis indicates the number of days for which the recipient monkey was under observation for malaria.

Spl. Indicates that malaria did not develop following splenectomy.

TABLE III.

PROTOCOLS OF EXPERIMENTS IN WHICH SPLEEN TISSUE WAS SUBINOCULATED AT INTERVALS OF 2 TO 14 DAYS FOLLOWING SPROZOITE INJECTION.

Recipient Monkey Number.	Donor Monkey Number	Time interval in days between sporozoite injection & removal of spleen for subinoculation.	Method of administration of sporozoites to donor. *	No. of infected mosquitoes used to infect donor £	Method of intraperitoneal subinoculation of spleen ()	Fraction of whole spleen subinoculated.	Results (+ malaria: - no malaria in		
							Donor	Blood Control @	Recipient. %
E-294	E-282	2	Intrasplenic (Thoraces)	79	TRANSPLANT	1	+	-	- (61)
E-296	E-264	4	Intrasplenic (Thoraces)	64	TRANSPLANT	1	+	-	- (61)
E-331	E-310	5	Mosquito Bite	65	SUSPENSION	½	+/	-	- (48)
E-105	E-95	6	Intravenous (Gut)	1	TRANSPLANT	1/6	+	-	- (43)
E-269	E-257	6	Mosquito Bite	100	TRANSPLANT	1	+	-	- (63)
E-266	E-253	7	Mosquito Bite	79	TRANSPLANT	1	+/	-	- (63)
E-284	E-256	7	Mosquito Bite	60	SUSPENSION	¾	+/	-	- (62)
E139	E-116	8	Mosquito Bite	5	SUSPENSION	1/6	+/	-	- (31)
E-99	E-147	10	Mosquito Bite	4	SUSPENSION	1/6	+	+	- (30)
E-96	E-144	12	Intravenous (Salivary Glands)	4	SUSPENSION	1/6	+	+	+
E-152	E-133	13	Intravenous (Salivary Glands)	2	SUSPENSION	½	+	+	+
E-169	E-134	14	Mosquito Bite	13	SUSPENSION	½	+	+	+

* The mosquito tissue in parenthesis indicates the source from which sporozoites were obtained, thus (salivary glands) signifies dissected glands while (thoraces) represents the entire thoracic contents including glands and (gut) means dissected mid-gut.

£ The number of infected mosquitoes was calculated by multiplying the actual number used by the index of experimental infection of the batch. The words intrasplenic and intravenous signify that the sporozoites were injected into the substance of the spleen and intravenously respectively.

() The term transplant signifies that the splenic tissue was subinoculated by transplantation and the word suspension means that a suspension of spleen tissue was prepared and subinoculated by injection intraperitoneally.

@ The blood control signifies a monkey which was subinoculated with blood removed immediately preceding the excision of the spleen tissue from the donor.

% The number in parenthesis indicates the period in days during which the recipient monkey was under observation for malaria.

/ These three monkeys were sacrificed during the incubation period, but as other monkeys on which the same batch of mosquitoes fed at the same time developed overt malaria they are recorded as positive.

TABLE IV.

PROTOCOLS OF EXPERIMENTS IN WHICH TISSUES OTHER THAN SPLEEN WERE SUBINOCULATED AT INTERVALS OF 5 TO 14 DAYS FOLLOWING SPOROZOITE INJECTION.

Recipient Monkey Number	Donor Monkey Number	Time interval in days between sporozoite injection and removal of tissue for subinoculation.	Method of administration of sporozoites to donor *	Number of infected mosquitoes used to infect donor £	Tissue used for subinoculation ()	Quantity of tissue used in subinoculation.	Results (+ malaria: - no malaria) Donor	Blood Control @	Recipient %
E-333	E-310	5	Mosquito Bite	65	Liver	Slice measuring 3" x 2" x ½"	+/	- (35)	- (35)
E-337	-do-	-do-	-do-	-do-	Bone Marrow	Marrow from Rt. Femur	+	- (35)	- (35)
E-336	-do-	-do-	-do-	-do-	Brain	¾ of Right Frontal Lobe	+	- (35)	- (35)
E-334	-do-	-do-	-do-	-do-	Lung	Lower lobe of right lung	+	- (35)	- (35)
E-335	-do-	-do-	-do-	-do-	Kidney	1/3 of right kidney (cortex & medulla)	+	- (35)	- (35)
E-332	-do-	-do-	-do-	-do-	Suprarenal	Left suprarenal (complete)	+	- (35)	- (35)
E-285	E-256	7	-do-	60	Liver	Slice measuring 3" x 2" x ½"	+/	- (50)	- (50)
E-289	-do-	-do-	-do-	-do-	Bone Marrow	Marrow from right femur	+	- (50)	- (50)
E-291	-do-	-do-	-do-	-do-	Brain	¾ of left Frontal Lobe	+	- (50)	- (50)
E-287	-do-	-do-	-do-	-do-	Lung	Lower lobe of left lung	+	- (50)	- (50)
E-288	-do-	-do-	-do-	-do-	Kidney	1/3 of right kidney (cortex & medulla)	+	- (50)	- (50)
E-286	-do-	-do-	-do-	-do-	Suprarenal	Left suprarenal (complete)	+	- (50)	- (50)
E-290	-do-	-do-	-do-	-do-	Pituitary	Pituitary (complete)	+	- (50)	- (50)
E-141	E-116	8	-do-	5	Liver	Slice measuring 1½" x ½" x ¼"	+/	- (116)	- (116)
E-140	-do-	-do-	-do-	-do-	Bone Marrow	All marrow from left femur	+	- (116)	- (116)
E-138	-do-	-do-	-do-	-do-	Brain	Slice measuring 1½" x 1" x ¼" from frontal lobe	+	- (116)	- (116)
E-142	-do-	-do-	-do-	-do-	Lung	Slice measuring 1" x 1" x ½"	+	- (116)	- (116)
E-143	-do-	-do-	-do-	-do-	Kidney	Slice measuring 1" x ½" x ¼" (Cortex & Medulla)	+	- (116)	- (116)
E-154	E-133	13	INTRAVENOUS (Salivary Glands)	2	Liver	Slice measuring 1½" x ½" x ¼"	+	+	
E-157	-do-	-do-	-do-	-do-	Bone Marrow	All marrow from left femur	+	+	- (221)
E-156	-do-	-do-	-do-	-do-	Brain	Slice measuring 1½" x 1" x ¼" from frontal lobe	+	+	+
E-153	-do-	-do-	-do-	-do-	Lung	Slice measuring 1" x 1" x ¼"	+	+	+
E-155	-do-	-do-	-do-	-do-	Kidney	Slice measuring 1" x ½" x ¼" (Cortex & Medulla)	+	+	+
E-158	-do-	-do-	-do-	-do-	Muscle	Slice measuring 1" x ½" x ¼"	+	+	+
E-168	E-134	14	Mosquito Bite	13	Brain	Slice measuring 1" x 1" x ½" from frontal lobe	+/	+	+

* The mosquito tissue in parenthesis indicates the source from which sporozoites were obtained, thus (salivary glands signifies dissected glands).

£ The number of infected mosquitoes was calculated by multiplying the actual number used by the index of experimental infection of the batch.

() A suspension in saline was prepared for subinoculation in all cases.

@ The blood control signifies a monkey which was subinoculated with blood removed immediately preceding the removal of the tissues from the donor.

% The number in parenthesis indicates the period in days during which the recipient monkeys and blood control were under observation for malaria.

/ These monkeys were sacrificed during the incubation period, but as other monkeys on which the same batch of mosquitoes fed at the same time developed overt malaria they are recorded as positive.

SECTION III.

THE MOSQUITO TRANSMISSION OF P.CYNOMOLGI TO M.MULATTA.

INTRODUCTION.

The mosquito transmission of P.cynomolgi to M.mulatta monkeys was a necessary preliminary to the collection of histological material for a search for exo-erythrocytic schizogony in the tissues, and to the series of experiments, which have been described in the previous section of this thesis, on the infectivity of the blood and tissues during the incubation period in sporozoite induced infections. The demand for a large number of monkeys, which had been injected with a heavy sporozoite dosage, necessitated the provision of a regular supply of large numbers of malaria infected mosquitoes. As no "colonised" mosquito was available in India at the time the adult mosquitoes used were reared from larvae collected from breeding places in the field. This meant that the mosquito transmission experiments could only be carried out in the spring and in the autumn, when larval breeding in the field was in progress.

I wish to relate an experience which taught me a severe lesson and which caused a good deal of quiet amusement to many people. We originally sited the insectary in the grounds of the Malaria Institute in Delhi. The authorities responsible for the malaria control of Delhi decided to carry out an extensive air spraying of the city with D.D.T. The planes came over, and appeared to me to concentrate their attack on the Malaria Institute. The mosquito mortality in my insectary was very heavy as there was

hardly a single survivor in the mosquito population. This necessitated a move to Karnal in the Punjab. Our troubles were not over as the D.D.T. persisted on the mosquito cages and we were forced to destroy them all and have new ones made. It taught me that D.D.T. and mosquito work are ill-wed companions. As the result of this experience I formed the opinion that entomologists when carrying out insect studies in the laboratory would be well advised to avoid entirely the use of D.D.T. in the same laboratory. In fact if I was ever called upon to organise an entomological section of a general laboratory I would certainly locate the insectary in a separate building miles out "in the blue" and well out of range of units responsible for malaria control in the field. Other difficulties were encountered in obtaining and maintaining a large supply of mosquitoes, infected with P.cynomolgi. These difficulties included the low index of experimental infection in certain batches, the high mosquito mortality during the sporogony cycle, and the failure in some cases to transmit the infection to the mammalian host. James' (1926) comment that "it is difficult to bring even a few numbers of a large brood of mosquitoes to a condition in which they will be successful transmitters of malaria" aptly expresses our difficulties in the mosquito transmission of P.cynomolgi.

An analysis of the experience of the mosquito transmission of P.cynomolgi to M.mulatta during the autumn of 1946, and the spring of 1946 and 1947 can be summarised as follows:-

1. Out of 33,712 mosquitoes which fed on gametocytic carriers only 5,619, i.e. 16 per cent were ultimately available

for the experimental transmission of the disease.

2. Only 126 batches out of 213 which fed on gametocytic carriers developed the infection, i.e., 58 per cent.

3. The index of experimental infection in the positive batches varied from 5 per cent to 100 per cent.

4. 97 monkeys out of a total of 147 in which transmission of the disease was attempted developed an infection, i.e., 65 per cent.

I propose to discuss some of the factors associated with the experimental mosquito transmission of P.cynomolgi, and for the sake of convenience in presentation they have been grouped under the following main heads:-

A. Factors relating to the transmission of P.cynomolgi from M.mulatta monkeys to mosquitoes.

B. Factors relating to the development of P.cynomolgi in mosquitoes.

C. Factors relating to the transmission of P.cynomolgi from mosquitoes to M.mulatta monkeys.

A. Factors relating to the transmission of P.cynomolgi from M.mulatta monkeys to mosquitoes.

1. Relationship to Anopheline Species.

Successful transmission to all the anopheline species which have been used in the present studies has been obtained. The development of the parasite up to the formation of sporozoites in the salivary glands has been observed in A.subpictus,

A.culicifacies, A.annularis, A.barbirostris, and A.hyrcanus. All attempts to transmit the infection to culicines failed.

The degree of susceptibility of different anopheline species to infection with P.cynomolgi appeared to vary considerably, being greatest for A.annularis and least for A.barbirostris. Table I illustrates the comparative susceptibility of the five anopheline species which were used in the present studies. The figures shown in the table have been computed from the totals of the different anopheline species in 19 positive mosquito batches in which the experimental conditions were identical for each individual mosquito batch.

It will be seen that the descending order of susceptibility to infection with P.cynomolgi is as follows:-

A.annularis, A.subpictus, A.culicifacies, A.hyrcanus and A.barbirostris.

2. Relationship to the Simian Source of Infection.

Particular monkeys did not possess any inherent ability to act as "good infectors" for mosquitoes. The majority of monkeys which were used on several occasions as the source of infection were infective to anophelines at some stage of the disease. The method by which the infection was introduced into the monkeys appeared to play no part in determining the transmission of the parasite to mosquitoes, as successful transmission was obtained equally readily in both sporozoite and blood induced infections.

3. Relationship to Gametocyte Prevalence.

The gametocyte prevalence in P.cynomolgi infections in M.mulatta monkeys occurs as a wave during the course of the primary appearance of asexual

forms and runs parallel with the wave of these forms during the course of the primary attack. They are found in greatest numbers at or about the peak of parasitaemia and rapidly decrease during the crisis. The gametocyte-asexual form ratio is fairly constant during the course of the primary attack being set at about 1 to 5 : 100.

No direct correlation was observed between gametocyte prevalence and infectivity to mosquitoes (Table II). It will be seen from the table that on several occasions successful feedings were obtained when no gametocytes were detected in blood films taken at the time of the mosquito feedings, and conversely, the transmission of the infection to mosquitoes was not obtained when the feeding was carried out on a monkey showing a high degree of gametocyte prevalence. These observations indicate that gametocyte prevalence per se can NOT be regarded as the criterion in the selection of a suitable monkey on which to feed a batch of mosquitoes.

4. Relationship to the stage of Infection.

The transmission of P.cynomolgi to mosquitoes was obtained at all stages of the primary attack of malaria in the mammalian host. It was found, however, that the index of experimental infection in the mosquito batches was higher in those which were fed before and after the parasite crisis, than in those fed during and immediately after the parasite crisis. An example of the results of serial feedings of different batches of mosquitoes on successive days during the course of the primary attack in the same monkey, illustrates the general

experience (Table III). Batches 124 and 127, fed on the day of the parasite crisis and two days after it respectively, show a lower index of experimental infection than batches 122 and 131, fed the day before it and 5 days after it respectively.

B. Factors relating to the Development of P.cynomolgi in mosquitoes.

1. The Duration of the Sporogony cycle and its relationship to Temperature.

The duration of the sporogony cycle was estimated in days from the date of feeding each mosquito batch on a gametocyte carrier to the date of first appearance of sporozoites on sample dissection. The samples for dissection were taken at random from the batch. The duration of the sporogony cycle varied from 13 days to 29 days. Maximum and minimum temperature readings were taken in the insectary at 8.a.m. and 4.p.m. daily. The range of variation in the duration of the sporogony cycle was related to the prevailing temperature conditions in the insectary during the development of the parasite in the mosquito host. Details of the duration of the sporogony cycle for different batches of mosquitoes are compared with the average mean temperature of the insectary during parasite development (Table IV). The sporogony cycle was 14 days when the average mean temperature in the insectary was 76.2°F (batch 34) and 28 days when the average mean temperature was 58.8°F (batch 84). The table also demonstrates that with a fall in the average mean temperature there was an increase in the length of the sporogony cycle.

2. Mosquito mortality, the intensity of mosquito infection and their relationship to temperature.

The heavy mosquito mortality was a constant

problem and observations showed that when the average mean temperature of the insectary rose above 78°F there was a steep rise in the mortality figures. The mortality was at a minimum when the average mean temperatures were between 60 - 70°F. The intensity of the mosquito infection as gauged by the numbers of oocysts in the stomach and the numbers of sporozoites in the salivary glands of the samples which were dissected as a routine from each batch, varied with the average mean temperature of the insectary. When this temperature was between 60 - 70°F the intensity of mosquito infection was low, but when it was between 70 - 76°F the intensity of mosquito infection greatly increased. This as will be shown later, is reflected in the results of attempted transmission of the disease to the mammalian host (Table V).

C. Factors relating to the transmission of P.cynomolgi FROM mosquitoes to M.mulatta.

1. Influence of Season.

The seasonal factor was extremely important in determining the success or otherwise of the mosquito transmission of the disease to the mammalian host. The results are recorded in Table V. This demonstrates the poor results which were obtained in March, 1946. It was also observed at this time that the intensity of mosquito infection was low, and that the maturation of the oocysts was slow. The mean temperature during this period varied from between 60° to 70°F.

2. Influence of Sporozoite dosage.

The observations on the influence of the sporozoite dosage on the successful transmission of P.cynomolgi to M.mulatta are summarised in Table VI.

It is felt that they do not show anything of particular interest as they are not based on experiments designed to determine the minimum infective dosage of sporozoites required to cause infection in the mammalian host. In fact the dosage in every group must be relatively large compared to that which occurs in human plasmodial infections. It is considered probable that such differences as are noted are an expression of the effect of temperature on the intensity of infection in the mosquitoes. Certainly included in the second group are several batches of mosquitoes which from the point of view of transmission proved sterile in early March,1946.

3. The influence of the source of the Sporozoites in the mosquito.

Table VII summarises the results of an analysis of the data designed to find out whether or not the source of the sporozoites in the mosquito played any part in the transmission of the infection to the mammalian host.

The chief point of interest lies in the observation that the injection of mosquito gut containing ripe oocysts succeeded as a method in the transmission of the disease. It is an effective answer to the belief that sporozoites undergo a stage of development in the salivary glands, and that this period of maturation in these glands is necessary for infectivity.

DISCUSSION.

The mosquito transmission of P.cynomolgi to M.mulatta is a relatively simple matter, but the provision on demand of a large supply of heavily infected mosquitoes is a problem. The difficulties

which were encountered in the work resembled those which James (1926) had to face at Horton during the early days of his studies on the mosquito transmission of human plasmodia to patients suffering from general paralysis of the insane. The ideal answer to the problem would be the use of a "colonised" vector maintained under controlled conditions of temperature and humidity. A.stephensi and A.fluviatilis would well repay study in this connection as both have been colonised. If, however, reliance had to be placed for the supply of mosquitoes on larval collections from the field it should be borne in mind that the optimum season for such work in the Punjab is from the second week in March to the third week in April. During this period A.annularis is a prolific breeder, the mosquito mortality is reasonably low, and the intensity of infection in the mosquitoes is high. This might be called the period of effective sporozoite transmission. During the weeks preceeding this period, experimental transmission of the disease is unsatisfactory and during the weeks following it the mosquito mortality is so high as to make it not worth while continuing such studies.

One of the chief difficulties which has to be overcome in providing large numbers of heavily infected mosquitoes is the provision and selection of suitable gametocyte carriers. I noticed early in the work that gametocyte prevalence by itself is no criterion on which to base the decision to feed a batch of mosquitoes on a particular monkey. Satisfactory results were obtained by feeding the mosquitoes on gametocyte carriers during the period of the acute attack of malaria, that is, during

the period of marked parasite prevalence. The results of feeding mosquitoes during the chronic stage of the infection were on the whole unsatisfactory even when there was a reasonable gametocyte prevalence. It should be noted, however, that feedings of mosquitoes carried out during and for a day or two after the parasite "crisis" resulted in a low index of experimental infection. The reason for this was not ascertained but the observation suggests the development at the "peak" of infection of an inhibitor to gametocyte conjugation. It may be that the inhibitor acts by inhibiting the exflagellation of the male gametocyte. The policy of feeding mosquito batches on gametocyte carriers during the acute attack necessitates a large supply of monkeys and foresight in preparing for anticipated demands.

I wish to draw attention to two other observations of interest, namely the different susceptibilities of the different anopheline species to infection with P.cynomolgi, and the fact that successful transmission was accomplished by the injection of mature oocysts. A.annularis was highly susceptible to infection with P.cynomolgi, whereas A.hyrcanus and A.barborostris were not so susceptible. This varying susceptibility of different anopheline species to plasmodial infection may be a factor in determining the transmission of human malaria by a particular vector in a region where there are several potential vectors. Great stress is rightly placed on the bionomics, particularly the feeding habits, of a vector species, but it seems to me that further study would be profitable along the lines of varying susceptibilities of different anopheline species to

infection with human plasmodia. The observation that the injection of mature oocysts induced an infection in the mammalian host lends very strong support to the view that the sporozoite reaches maturity in the oocyst and does not go through a further period of development in the salivary glands of the mosquito.

The many difficulties which were experienced in the experimental mosquito transmission of P.cynomolgi to M.mulatta certainly serve to demonstrate the many hazards to which plasmodia have been and are exposed in their struggle for existence.

TABLE I.

Table illustrating the comparative susceptibility of different Anopheline species to P.cynomolgi.

Anopheline Species.	Total number examined.	Total number infected (Oocysts or sporozoites).	Percentage number infected. (Oocysts or sprozoites.)
A.annularis	87	45	51.7
A.subpictus	42	15	35.7
A.culicifacies	32	7	21.8
A.hyrcanus	49	5	10.2
A.barbirostris	12	1	8.3

TABLE II.

Table illustrating the lack of relationship between gametocyte prevalence and infectivity for mosquitoes to P.cynomolgi.

Mosquito Batch Number.	Gametocyte prevalence on day of mosquito feeding per 10,000 per red cells.	Index of experimental infection oocysts or sporozoites expressed as a percentage.
39	100	0
24	50	0
21	40	83
162	40	100
54	30	0
66	30	5
37	20	0
110	20	0
163	20	100
171	20	0
173	20	66
41	10	0
50	10	7
56	10	0
164	10	0
112	V.scanty	57
166	"	0
64	"	75
97	V.V.scanty	0
102	"	0
113	"	5
120	"	20
80	"	33
122	"	50
139	"	71
152	"	83
155	"	100
160	"	100
104	Nil	0
105	"	8
118	"	20
107	"	40

TABLE III.

Table illustrating the results of serial feedings of different mosquito batches of the same anopheline species on the same monkey E-175 during the course of the primary attack.

Mosquito batch number.	Day of mosquito feeding relative to 1st appearance of parasites.	Count of asexual parasites per 10,000 red cells.	Count of gametocytes per 10,000 red cells	Index of experimental infection of mosquito batch (Oocysts or sporozoites) Per cent
118	3	80	-	20
120	4	140	VVS	20
121	5	450	VVS	25
122	6	810	VVS	50
124	7	1790	VVS	9
127	9	520	VVS	10
131	12	200	VVS	75
132	13	400	VVS	25
134	14	250	VVS	10

TABLE IV.

Table illustrating the duration of the Sporogony Cycle of P.cynomolgi and its relationship to temperature.

Batch Number.	Average maximum temperature in degrees Fahrenheit.	Average minimum temperature in degrees Fahrenheit.	Average mean temperature in degrees Fahrenheit.	Date of feeding on a gametocyte carrier.	Date of first appearance of sporozoites on dissection.	Duration of sporogony cycle in days.
34	86.2	66.2	76.2	26. 9.45	10.10.45	14
35	87.4	66.5	76.9	27. 9.45	11.10.45	14
36	87.4	66.5	76.9	27. 9.45	11.10.45	14
53	88.4	63.4	75.7	10.10.45	24.10.45	14
55	87.4	62.9	75.2	11.10.45	26.10.45	15
60	87.2	63.2	75.2	15.10.45	29.10.45	14
64	85.3	62.5	73.9	19.10.45	1.11.45	13
65	84.2	59.8	72.0	20.10.45	5.11.45	16
68	82.9	48.5	65.7	26.10.45	10.11.45	15
74	79.5	46.4	62.9	8.11.45	2.12.45	24
75	78.0	45.0	61.5	9.11.45	8.12.45	29
76	77.8	44.4	61.1	12.11.45	8.12.45	26
79	77.4	43.9	60.7	14.11.45	8.12.45	24
80	76.9	43.8	60.4	15.11.45	9.12.45	24
83	75.3	42.5	58.9	16.11.45	15.12.45	29
84	75.1	42.4	58.8	17.11.45	15.12.45	28

TABLE V.

Table illustrating the influence of the season on the Mosquito transmission of P.cynomolgi to M.mulatta.

Month	Year	Range of Mean Temperature	No.of monkeys to which transmission was attempted. £	No. of monkeys which developed malaria.	No. of monkeys which failed to develop malaria	Percentage of monkeys which developed malaria.
October	1945	72° - 78°F.	21	14	7	66.6%
November	1945	64° - 72°F	7	5	2	71.4%
* Early March	1946	60° - 70°F	30	7	23	23.3%
April	1946	70° - 78°F	23	18	5	78.2%
/ Late March	1947	72° - 76°F	29	25	4	86.2%
April	1947	72° - 80°F	29	28	1	96.5%

£ Monkeys in which transmission was attempted by injection of mosquito guts containing oocysts have been excluded.

* The majority of cases attempted between 8th - 20th March.

/ All cases attempted between 28th - 31st March.

TABLE VI.

Table illustrating the influence of the Sporozoite Dosage on the successful transmission of P.cynomolgi to M.mulatta.

Infection Produced by Mosquito Bite in all Cases.

Sporozoite dosage expressed as number of infected mosquitoes used in transmission £	Number of monkeys to which transmission was attempted.	Number of monkeys which developed malaria.	Number of monkeys which failed to develop malaria.	Percentage of monkeys which developed malaria.
1 - 9	29	22	7	75.8%
10 - 99	32	19	13	59.3%
100 or more	5	5	0	100%

£ The number of infected mosquitoes was calculated by multiplying the actual number used by the index of experimental infection of the batch.

TABLE VII.

Table illustrating the influence of the source of Sporozoites in the Mosquito on the successful transmission of P.cynomolgi to M.mulatta.

Source of sporozoites in the mosquitoes. £	Number of monkeys to which transmission was attempted.	Number of monkeys which developed malaria.	Number of monkeys which failed to develop malaria.	Percentage of monkeys which developed malaria.
Salivary glands (Mosquito bite).	66	46	20	69.6%
Salivary glands (Intravenous)	14	11	3	78.5%
Thorax inluding salivary glands (Intradermal and intrasplenic)	20	8	12	40%
	* 7	6	1	85.7%
Gut containing ripe oocysts (Intravenous).	8	2	6	25%
Thorax and Abdomen including salivary glands and gut. (Subcutaneous)	20	19	1	95%

£ The words in parenthesis indicate the route by which the mosquito tissue containing sporozoites was inoculated.

* These figures represent the results for the month of April, i.e., the month of optimum transmission see Table V.

SECTION IV.

SOME OBSERVATIONS ON THE COURSE OF SPOROZOITE INDUCED P.CYNOMOLGI INFECTIONS IN M.MULATTA.

INTRODUCTION.

The observations to be described in this section, on the course of P.cynomolgi in M.mulatta, were carried out during the course of the Mammalian Malaria Enquiry. Although they were incidental to the main purpose of that enquiry, namely the search for pre-erythrocytic forms of the parasite, they are nevertheless of interest and would certainly form a useful baseline for chemotherapeutic studies on P.cynomolgi in M.mulatta. The analysis is based on a study of 108 sporozoite infected monkeys. A similar study has been reported by Wolfson and Winter (1946), but in their series the observations made dealt almost entirely with the course of the disease in blood induced infections. The present report is therefore, supplementary to that of Wolfson and Winter.

I propose to deal in turn with the incubation period, the period of the acute attack, deaths, the length of the disease and relapses. For the sake of convenience the tables have been placed at the end of the section.

RESULTS.

1. Incubation Period.

(a). In sporozoite induced infections.

The incubation period has been considered as that period in days from the time of inoculation of sporozoites to the day of first appearance of parasites in thick films of the peripheral blood. The day of inoculation of sporozoites was considered

as the first day.

The incubation period varied from 10 to 31 days with a mean of 14.7 days and a standard deviation of 5.8 days. The great majority of the cases had an incubation period of 12 to 17 days as is shown in the table of frequency distribution (Table 1).

Two further cases are of interest because they had a protracted incubation period. In the first case (E159) sporozoite inoculation was effected by 11 infected mosquitoes. Splenectomy was performed 38 days after sporozoite inoculation and overt malaria occurred 12 days later, that is, there was an incubation period of 50 days. In the second case (E-125) the salivary glands of two infected mosquitoes were injected intravenously. Splenectomy was performed 56 days after sporozoite inoculation and overt malaria occurred 52 days later, that is, the incubation period was 108 days. (Table II). The argument that natural mosquito transmission might have occurred weeks after the experimental transmission is not considered to be valid as these monkeys were under observation during a period when natural transmission would not be possible, namely, late autumn into the winter. It seems more likely that the sporozoites were developing slowly in the liver cells during these long incubation periods, and that the invasion of the erythrocytes was delayed. The fact that 52 days elapsed after splenectomy in case E-125 lends support to this view.

The influence of the sporozoite dosage on the incubation period was not marked but when very large numbers of infected mosquitoes were used the standard deviation was less pronounced (Table III). It will be noticed from the table that the infection was produced by mosquito bite in all cases.

(b). In blood induced infections.

It may be stated in general terms that my experience was the same as that recorded by Wolfson and Winter (1946), namely, that the length of the incubation period is inversely related to the number of blood parasites injected; if these are very numerous, parasites may be visible in the monkey's blood within a few hours of the time of injection.

2. Period of the Acute Attack.

No differences were detected during the period of parasite prevalence in sporozoite induced and blood induced infections. I propose therefore to outline my experience with sporozoite induced infections.

The period of the primary attack can be divided into the period of the acute rise, during which parasites are multiplying and increasing in number, the crisis and the period of parasite decline. The length of the primary attack is sometimes difficult to determine as it sometimes happens that there is a short secondary rise in parasite prevalence within a day or two after the fall to levels at which parasites cannot be counted. It is necessary, therefore, to adopt an arbitrary definition for the length of the primary attack. For the purposes of this analysis it has been defined as the number of days from the onset of parasitaemia until a week after parasites are no longer countable and have remained uncountable.

(a). Length of primary attack.

In 83 cases the length of the primary attack varied from 7 days to 19 days with a mean of 11.23 days (Table IV). The frequency distribution is shown in the table.

(b). Length of the "acute rise" of parasitaemia.

In 94 cases the length of the acute rise of parasitaemia varied from 4 days to 11 days with a

mean of 6.74 days (Table V). The frequency distribution is shown in the table.

(c). The height of the peak of the primary wave of parasitaemia.

In 95 monkeys the mean height of the peak of parasite prevalence during the primary attack was 810 parasites per 10,000 red cells. The cases have been grouped for convenience in counts at intervals of 100 parasites. The frequency distribution is shown in Table VI. It will be noticed that there is a wide range of variation in the degree of parasite prevalence at the peak of infection, varying from less than 1% of red cells parasitized up to 30% of red cells parasitized.

3. Deaths.

In the group of sporozoite induced infections with P.cynomolgi one death out of 108 cases occurred. This was in a monkey which was inoculated with 114 infected mosquitoes subcutaneously. It died at the peak of parasitaemia of the second relapse 47 days after the onset of the clinical infection. At the time of death 25% of the red cells were parasitized but there was no haemoglobinuria. This means that the mortality rate was less than one per cent.

4. Length of the Disease.

It is a matter of extreme difficulty to determine the length of infection with malaria because the tests of cure, namely superinfection with the homologous strain or splenectomy, irrevocably interfere with further observations. Further, reliance cannot be placed on observations based on the examination of thick films of the peripheral blood as prolonged latent periods often occur. In the present series 13 sporozoite infected monkeys were under observation for more than

3 months. The length of time during which parasites were observed in thick smears varied from 21 to 262 days with a mean of 184.

A test of cure was carried out by splenectomy in five cases and the results are summarised in Table VII. It will be seen that infection was still present in 3 out of the 5 from 13 - 17½ months after the commencement of the disease. The remaining two were apparently cured 13 months after the onset of the disease.

It would seem therefore that in P.cynomolgi infections in M.mulatta, when induced by sporozoite inoculation, the length of the disease varies from monkey to monkey. In some cases spontaneous cure was effected about a year from the date of infection, but in other cases infection was still present 1½ years after the commencement of the disease.

5. Relapses.

24 monkeys with sporozoite induced P.cynomolgi infections were observed for 9 weeks or more to determine the frequency of parasitic relapse. In five cases a parasitic relapse was observed, that is in 21% of cases. In every case the parasitic relapse occurred within 8 weeks of the primary attack, and resembled that attack more or less. In some cases the parasitic prevalence was higher in the relapse than in the primary attack and in others the reverse was the case.

SUMMARY.

The following is a summary of the observations made on 108 M.mulatta monkeys which suffered from P.cynomolgi infections produced by sporozoite inoculation:-

1. The incubation period varied from 10 - 31 days with a mean of 14.7 days.

2. Two cases with protracted incubation periods of 50 and 108 days respectively are recorded.
3. The length of the primary attack (83 cases) varied from 7 - 19 days with a mean of 11.23 days.
4. The length of the "acute rise" of parasitaemia (94 cases) varied from 4 - 11 days with a mean of 6.74 days.
5. The height of the peak of the primary wave of parasitaemia (95 cases) varied from less than 1% to 30% of parasitized erythrocytes with a mean of about 8% of parasitized red cells.
6. One case out of 108 observed died from the effects of infection with P.cynomolgi.
7. Spontaneous recovery,as judged by the effects of splenectomy, was observed in 2 cases 13 months from the time of original infection. In 3 others the disease was still present 13 - 17½ months after the original attack of malaria.
8. Relapses occurred in 21% of cases observed (19 cases) during a period of several months observation after the primary attack. In every case the first relapse took place within 8 weeks from the primary attack.

TABLE I.

Frequency distribution of days in incubation period of sporozoite induced P.cynomolgi infections in M.mulatta.

Length of incubation period in days.	10	11	12	13	14	15	16	17	18	19
Number of Cases.	1	4	12	18	18	24	10	10	5	1

20	21-29	30	31
1	0	1	1

Mean of 106 cases 14.7 days ± 5.8 days.

TABLE II.

Table illustrating the occurrence of Protracted Incubation Periods of P.cynomolgi in M.mulatta following Sporozoite Inoculation.

Monkey Number.	Method of administration for sporozoites. *	Number of infected mosquitoes used. £	Period in DAYS after sporozoite inoculation when splenectomy was carried out.	Incubation period in DAYS. /	REMARKS
E-159	MOSQUITO BITE.	11	38	50	Overt malaria occurred 12 days after splenectomy
E-125	INTRAVENOUS (Salivary glands).	2	56	108	Overt malaria occurred 52 days after splenectomy

* The mosquito tissue in parenthesis indicates the source from which the sporozoites were obtained.

£ The number of infected mosquitoes was calculated by multiplying the actual number used by the index of experimental infection of the batch.

/ Day of inoculation of sporozoites counts as 1.

TABLE III.

Table illustrating the Influence of the Sporozoite Dosage on the Incubation Period of P.cynomolgi in M.mulatta.

INFECTION PRODUCED BY MOSQUITO BITE IN ALL CASES.

Sporozoite Dosage expressed as Number of infected mosquitoes used to infect donors. £	Number of monkeys observed	INCUBATION PERIOD IN DAYS. Range	Mean	Standard Deviation
1 - 9	22	12 - 19	16.0	2.19
10 - 99	19	13 - 20	15.58	1.59
100 or more.	5	13 - 15	14.0	0.9

£ The number of infected mosquitoes was calculated by multiplying the actual number used by the index of experimental infection of the batch.

TABLE IV.

Frequency distribution of days of the length of the primary attack of sporozoite induced P.cynomolgi infections in M.mulatta.

Number of days.	7	8	9	10	11	12	13	14
Frequency	8	8	10	10	14	10	8	2

Number of days.	15	16	17	18	19
Frequency	4	2	3	3	1

Mean of 83 cases = 11.23 days.

TABLE V.

Frequency distribution of days in the acute rise of sporozoite induced P.cynomolgi infections in M.mulatta.

No. of days to peak	4	5	6	7	8	9	10	11
No. of cases.	6	18	24	20	13	8	3	2

Mean of 94 cases = 6.74 days.

TABLE VI.

Frequency distribution of the height of peak of the primary wave of parasitaemia in sporozoite induced P.cynomolgi infections in M.mulatta.

Parasite count at peak per cent of red cells.	less than 1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	15-20	20-25	30
No. of cases.	4	4	2	10	10	11	7	8	11	3	2	5	5	2	2	2	3	3	1

TABLE VII.

Demonstrating the persistence of sporozoite induced P.cynomolgi infections in M.mulatta when tested by splenectomy.

Monkey Number	Length of parasitaemia	Length of free period.	Total time between infection and splenectomy.	Result of Splenectomy.
E-119	8½ months	8 months	16½ months	Relapse
E-145	4½ months	13 months	17½ months	Relapse
E-243	4 months	9 months	13 months	No Relapse
E-270	3 months	10 months	13 months	Relapse
E-301	5 months	8 months	13 months	No Relapse

SECTION V.

STUDIES ON THE EFFECT OF DIET ON THE COURSE OF EXPERIMENTAL MALARIA IN MONKEYS.

INTRODUCTION.

The association of malaria, economic stress, and malnutrition has interested malariologists, puzzled economists, provoked departments of the Government of India, and even caused anxiety in Whitehall. It certainly has produced a voluminous literature which I do not propose to review. The problem is difficult to study in the field because of the large number of variables involved, climatic, economic and epidemiological, each so interwoven with the other that a picture of amazing complexity is the outcome. Hackett (1937) aptly sums up the position when he says "the absence of experimental data makes the discussion of these questions a mere battle of opinion".

The production of experimental malaria in monkeys provides a method of tackling the problem. Monkeys kept in a uniform environment can be infected with constant amounts of various malarial parasites, and the course of the infection in animals given different kinds of diet observed. Investigations on experimental malaria in monkeys, and the effect on monkeys of various kinds of defective diets, were carried out side by side at Coonoor, thus affording an opportunity to study one aspect of the question at issue.

EXPERIMENTAL.

I am indebted to Captain R.Passmore, I.M.S., for the information contained in this and the subsequent sub-section. They have been included in

order to provide the background to the malaria experiments.

For convenience in binding the tables have been grouped at the end of the section.

The species of monkey used in the experiments was Macacus radiatus (S.sinicus), the common South Indian monkey. The animals were obtained from dealers and fed liberally and acclimatized for at least a month before being put on experimental diets. Two groups of animals, A and B, fed on two different types of diet, were used for the experiment. Diet A resembled a good human lacto-vegetarian diet, as consumed by certain groups in North India. It was based on whole wheat and contained liberal amounts of pulses, vegetables, fruits and milk. Diet B resembled the kind of diet consumed by poor rice-eaters all over India, which has been described by Aykroyd, Krishnan, Passmore and Sundararajan (1940). The main ingredient was parboiled milled rice, and only small amounts of pulses, vegetables and fruits, and no milk, were included.

The approximate composition of the diets is shown in Tables I and II. The monkeys on diet A usually consumed all the fruits and vegetables and practically all the milk. The wheat part of the ration, given in the form of a chappati, was not always completely eaten - a variable amount, usually about one-third, being left unconsumed. The ingredients of the rice diet B were cooked together and offered ad lib. to the animals.

State of Nutrition of the Groups.

The animals used for the malaria experiments were taken from larger groups fed on these diets. Striking differences in the health and well-being of

the two groups were observed, the advantage of belonging to group A being shown by a number of criteria. The monkeys on diet A put on weight and in general remained in excellent condition. Certain animals have thriven for over 2 years on this diet. While some deaths from diarrhoea and various intercurrent infections occurred in Group A, the average survival period in this group was more than twice as long as in group B.

Most animals fed on the rice diet lost weight steadily; a few maintained their original weight for several months. Some went downhill rapidly and died within 6 or 8 weeks. The majority, however, survived for 6 to 12 months in an impaired state of health. They usually died after suffering for several weeks from diarrhoea associated with inflammatory or degenerative changes in the small intestine. The incidence of attacks of diarrhoea in group B was about three times as great as in group A. Conjunctivitis and xerosis, suggestive of vitamin A deficiency, were observed in a few animals. A few showed signs attributable to mild scurvy. Post-mortem examinations revealed a whole series of pathological lesions, in general absent in group A.

The monkeys in group A, when out at exercise, were active and playful climbing all over the enclosure. Group B, on the other hand, sat about listlessly taking little interest in their surroundings, their most energetic pastime being the picking of each other's pelts. On approaching the enclosure

it was usually possible to distinguish from a distance of about 25 yards which group was out at exercise, from the behaviour of the animals.

The two groups were, thus, clearly differentiated as regards health, activity and the incidence of certain diseases, and the difference was due solely to diet. One group was in a relatively good state of nutrition, the other in a wretched state of nutrition. Apart from the diet, the environment of the groups was exactly the same.

Malaria Infections.

All the animals when infected had been fed on the respective diets for periods ranging from 3 to 18 months. Two series of experiments were carried out. In one, 5 monkeys from each group were infected with Plasmodium cynomolgi. In the other, 7 from group A and 8 from group B were infected with Plasmodium knowlesi. Blood obtained from infected monkeys of the same species was injected intraperitoneally. The infecting doses of P.cynomolgi and P.knowlesi were approximately 75 and 50 million parasites respectively.

During the period of infection, blood films were taken four times daily, at 8.a.m., 12 noon, 4.p.m., and 8.p.m. The number of parasitized red cells per 10,000 was counted, and the prevalence of asexual forms and gametocytes noted. The incubation period, and the duration of the attack as indicated by the return of the parasite count to a low figure, were compared in the two groups.

RESULTS.

Monkeys infected with P.cynomolgi.

The results are summarized in Table III. In neither group, did any animal show clinical signs of

illness. The prevalence of asexual forms was low in both groups, the maximum being 150 per 10,000 red cells, in one monkey in group A. The average maximum parasite prevalence was 20 and 80 per 10,000 in groups A and B respectively. Gametocytes were found only occasionally in the films from both groups. There was no difference in the length of the incubation period. Ten days after incubation, parasites were scanty in all the blood films.

Monkeys infected with P.knowlesi.

The results are summarized in Table IV. All the monkeys showed signs of illness at the height of the infection. They lost their appetites and most of them sat in the corner of their cages in the characteristic position of the sick monkey, with the hands behind the head. Two monkeys from each group became extremely ill. Of these, the two from group B died, as did one of the animals from group A. The second monkey in group A, which became very ill, recovered. In each case, death occurred 2 to 3 days after the period of maximum parasite prevalence had been reached. The average maximum parasite count was 1,830 and 1,500 per 10,000 red cells in groups A and B respectively. Gametocyte prevalence was low and similar in both groups; the maximum count was 50 per 10,000 red cells, which occurred in the infant monkey, No.76a. The average incubation period was 6½ days in both groups, and the average duration of the primary attack was substantially the same, viz., 8.5 days in group A and 7.5 days in group B.

RELAPSE AFTER SPLENECTOMY.

Four of the monkeys infected with P.knowlesi were splenectomized 6 to 8 weeks after recovery from the primary attack. Splenectomy invariably produces

relapse in such circumstances. The monkeys used for this experiment were Nos. 68, 71, 78 and 80, i.e., two from each diet group. Nos. 71 and 80 died in 21 and 14 days respectively, the maximum parasite count being about 2,000 per 10,000 in each case. Nos.68 and 78 survived, the parasite count being 400 to 500 at its highest level. The results of relapse following splenectomy were thus similar in monkeys belonging to both diet groups.

If the blood of a debilitated and malnourished individual contained more sexual forms than the blood of a well-nourished individual in the same stage of the disease, a partial explanation would be provided of the occurrence of fulminant epidemics in times of scarcity and famine. The present experiments, as far as they go, suggest that this possibility can be excluded.

DISCUSSION.

Although considerable variation was observed in the intensity of the malarial infections in individual animals, it has been clearly demonstrated that primary infections with P.cynomolgi or P.knowlesi did not differ significantly in two groups of monkeys, one well-nourished and the other in a state of malnutrition. Malnutrition was the result of a diet sufficient in quantity but poor in quality.

The experiment concerned only one aspect of the problem of the relation between malaria and nutrition. It confirms general experience that the severity of first attacks of malaria in individuals and groups is not mitigated by a good state of nutrition. But how far the general reaction of a community to malarial infection, as shown by the tendency to re-

lapse, the spleen rate, the amount of anæmia and the mortality rate from fever is influenced by the dietary factor, remains a subject for speculation.

SUMMARY.

The course and severity of primary attacks of malaria in monkeys infected with P.cynomolgi or P.knowlesi were unaffected by the differences in the state of nutrition of the monkeys previous to infection.

TABLE I.

DIET A, BASED ON WHOLE WHEAT AND CONTAINING LIBERAL AMOUNTS OF MILK, VEGETABLES AND FRUIT.

Atta (whole wheat) ..	100 gm. per monkey per day.
Pulse	15 " " " " "
Whole Milk	150 " " " " "
Butter or ghee.. ..	10 " " " " "
Root vegetables ..	40 " " " " "
Other vegetables (raw) ..	40 " " " " "
*Fruit	20 " " " " "

* Given on Monday, Wednesdays and Fridays only, about 50 gm. at a time.

Pulse: Bengal gram, black gram, green gram and dhal arhar - equal parts made into flour.

Root Vegetables: Potato, carrot, sweet potato, turnips.

Other Vegetables:

Sunday	.. Bitter gourd.
Monday	.. Cabbage
Tuesday	.. Cucumber or calabash cucumber.
Wednesday	.. Lady's Finger.
Thursday	.. Brinjal
Friday	.. Pumpkin.
Saturday	.. Green plantain.

Fruit: Plantain, mango, orange, apple or papayya.

The atta, pulse, whole milk and butter or ghee were mixed and made into a chappati. Half the chappati was given at 9.a.m. and the other half at 3.p.m. Root vegetables were given in the morning and the other vegetables (raw) and fruit in the evening.

TABLE II.

DIET B, BASED ON MILLED RICE AND CONTAINING SUPPLEMENTARY FOODS IN SMALL QUANTITIES.

Parboiled milled rice ..	100 gm. per monkey per day
*Vegetable	10 " " " " "
*Pulse	7 " " " " "
Gingelly oil	0.5 cc." " " "
Coconut oil	0.25 " " " " "
*Salt	0.5 gm." " " "
*Chillies (powder) ..	0.25 " " " " "
*Tamarind	0.25 " " " " "

* These were made into a soup and mixed with the cooked rice; gingelly oil and coconut oil were added.

Half the above quantity was given at 9.a.m. and the other half at 3 p.m. The amount of food supplied was always somewhat in excess of requirements.

Diet A was superior to diet B in respect of nearly all the important food factors. The latter was particularly deficient in vitamins A and C and calcium.

TABLE III.

THE EFFECT OF MALARIAL INFECTION (P.CYNOMOLGI) ON MONKEYS FED ON A WELL-BALANCED DIET (A) AND ON AN ILL-BALANCED RICE DIET (B).

Animal No.	Weeks on Diet.	Change in weight during period on experimental diets preceding infection (oz.)	Maximum parasite count. Parasitized cells per 10,000 red cells.
		Group A. Animals on well-balanced diet.	
22	45	+24	11
26	31	+13	84
23	49	+16	48
27	35	+31	150
25	36	+22	6
		Average ..	60
		Group B. Animals on ill-balanced diet.	
29	35	- 4	40
18	45	+ 5	12
15	49	+ 9	42
13	51	+15	Scanty.
28	22	- 3	10
		Average ..	20

Each animal received 2.0 - 3.0 ml. of blood from an infected monkey intraperitoneally. Ten days after infection only occasional parasites could be found in all cases.

TABLE IV.

THE EFFECT OF MALARIAL INFECTION (P.KNOWLESI) ON MONKEYS PREVIOUSLY FED ON A WELL-BALANCED DIET (A) AND ON AN ILL-BALANCED RICE DIET (B).

Animal No.	Change in weight during 12 weeks previous to infection (oz)	Incubation Period (days)	Maximum parasite count. Parasitized cells per 10,000 red cells	Length of infection (days)	Remarks.
		Group A. Animals on well-balanced diet.			
75	+ 5	6	2,340	5	Died
76	+ 2	6	2,520	8	Lactating mother.
76a	?	5	2,870	11	Infant, partially at breast.
77	+ 0	8	1,700	9	
78	+14	8	890	8	
79	+15	5	1,500	8	
80	+16	8	1,030	8	
Average	+10	6.4	1,830	8.5	Survivors only.
		Group B Animals on ill-balanced diet.			
64	-20	6	1,440	12	Died
66	-14	8	1,250	11	Died
68	+ 1	6	1,050	6	
69	+ 2	7	2,400	10	
70	-14	6	750	7	
71	- 6	6	3,120	7	
73	+ 2	6	1,300	7	
74	- 2	7	710	8	
Average	- 6.4	6.5	1,500	7.5	Survivors only.

Each monkey received 50 million parasites intraperitoneally. The length of infection is taken as the number of days during which the parasite count rose above 50 per 10,000 red cells.

The animals had been on their respective diets for 12 weeks previous to infection. During this period, four animals on the ill-balanced rice diet died.

SECTION VI.

HUMORAL AGENCIES IN DEFENCE AGAINST MALARIA.

The Chicago school of protozoologists have clearly demonstrated the importance of the cellular mechanism of defence against malaria (Cannon and Taliaferro 1931; Taliaferro and Cannon 1936; Taliaferro and Mulligan 1937). These workers, however, attributed the high specificity of acquired immunity to malaria to the presence of antibodies. Coggeshall and Kumm (1937; 1938) obtained proof of the existence of protective antibodies in the blood of monkeys which had acquired a high degree of immunity to infection. These authors' found that large doses of immune serum must be given to demonstrate a protective effect. They concluded, therefore, that antibodies against malaria were present in low concentration in the peripheral blood. Taliaferro and Cannon (1936) described a striking regional concentration in the Billroth cords of the spleen at the commencement of the crisis in infections with P.brasilianum in monkeys. This suggested the possible occurrence of protective antibodies at high concentration in the spleen.

The series of experiments described in this section were undertaken with a view to determining the effects of immune serum and spleen extract in controlling the course of P.knowlesi and P.cynomolgi infections in monkeys.

For sake of convenience of reference the monkeys from which the sera and spleen extracts were obtained have been classified in the protocols as

acute, subacute, chronic and superinfected. The definitions of these terms are as follows:-

1. Acute - represents a donor at or near the peak of the primary attack.
2. Sub-acute - represents a donor after the "crisis" of the primary attack, but before a state of chronicity or latency had been established.
3. Chronic - represents a donor after the primary attack had been overcome by natural or therapeutic means, and a state of chronicity or latency had been established.
4. Superinfected - represents a donor superinfected by homologous superinfection on one or more occasions during the chronic or latent stage of the infection. Superinfections were made for the dual purpose of demonstrating effective acquired immunity and of maintaining infection in donor animals. No deliberate attempt was made to increase the potency of immune serum by repeated superinfections with exceptionally large doses of homologous parasites.

A summary of the previous histories of the donor monkeys is given in an Appendix at the end of the section. As far as possible the donor monkeys were bled as required to obviate storage of the serum for lengthy periods.

Spleens for the preparation of extracts were removed from donor animals either by splenectomy or

after sacrifice by exsanguination. After removal the spleen was weighed, cut in pieces and transferred to a wide-mouthed bottle containing a measured quantity of sterile normal saline solution and a number of heavy glass beads. Care was taken to transfer all spleen fluids to the bottle. A suspension of spleen was made by vigorous shaking with glass beads.

Injections of sera and spleen extracts were made intraperitoneally in every case. In some experiments treatment was commenced on the day following the injection of the infecting dose of parasites, which in others, it was withheld until after parasites had appeared in the peripheral blood.

The effects of treatment were evaluated by a study of the course of infection in treated animals and compared with that in untreated controls. Blood films were taken daily at 8.a.m. 12.noon. 4.p.m. and 8.p.m. The average of the four daily parasite counts was taken as an index of the parasite prevalence for that day. These figures have been expressed in the protocols as percentages of infected red cells.

RESULTS OF EXPERIMENTS WITH HOMOLOGOUS SERA.

1. S.sinicus infected with P.knowlesi.

(a). Untreated.

Infections with P.knowlesi were carefully studied in 25 untreated sinicus monkeys. Individual animals varied considerably in their susceptibility to this infection. The least susceptible of the series showed only 5 per cent of parasitized

erythrocytes on the day of maximum parasite prevalence, while the most susceptible died with over 70 per cent of the red cells infected. The average of the highest daily counts for the series was 17 per cent of parasitized erythrocytes. Seven of these 25 monkeys (28 per cent) succumbed to the infection. The severity of the resultant infections in these monkeys appeared to be entirely independent of the number of parasites injected. The number of parasites inoculated varied from 5 million to 500 million in individual monkeys. The most severe infection observed in this series followed the injection of 5 million parasites, and the mildest infection was caused by the injection of 500 million. The incubation period was not appreciably shorter in monkeys inoculated with larger, as compared to smaller, numbers of parasites within the limits stated.

(b). Treated.

Eighteen sinicus monkeys infected with P.knowlesi were treated with homologous serum. Details of these experiments are given in Table I. On the whole, these 18 monkeys suffered less severely than the 25 untreated monkeys. Only two of the 18 animals (11 per cent) succumbed to the infection, and the average of the highest daily parasite counts was 11 per cent of parasitized erythrocytes. The intensity of infection varied greatly in individual animals, and for this reason it was difficult to assess the value of serum treatment. In only two instances (monkeys 32 and 51) were the primary attacks of less intensity and of shorter duration than in the mildest infection in the untreated group.

Monkeys 19, 30, 66, 77 and 78 received homologous serum from donors bled during the primary acute

attack. No definite evidence was obtained that the administration of serum modified the course of the initial infection in any of these animals.

Monkeys 32 to 39, inclusive, were treated with serum obtained from an exceptionally large monkey, which was sacrificed shortly after the "crisis" of the primary acute attack. It was thought that antibodies might be at high concentration in the blood stream at this stage of the infection. Monkeys 32 and 33 received serum treatment throughout the incubation period and after parasites appeared in the peripheral blood. One of these monkeys (32) developed a very mild attack of short duration, while the other (33) developed a moderately severe attack. Monkeys 34, 35 and 36 were treated as soon as parasites had been detected in the peripheral blood, and all three developed comparatively mild infections. Monkeys 37, 38 and 39 received no treatment until over 5 per cent of the red cells were parasitized. These three monkeys developed somewhat heavier infections than those in which treatment was commenced earlier, and one succumbed to the infection.

Monkeys 21 and 25 were treated with chronic serum, and monkeys 51, 55 and 62 with superinfected serum. In only one of these five monkeys (51) was there definite evidence of the beneficial effects of serum treatment. Of the remaining four monkeys, three developed comparatively mild infections, and one (21) developed a fairly severe infection from which it recovered.

It was hoped that sinicus monkeys, being less susceptible to the pathogenic effects of P.knowlesi than S.rhesus, would prove to be more

suitable for experiments of this kind. Unfortunately, the wide variations in the intensity of infections in individual untreated monkeys largely obscured the effects of immune serum treatment. Nevertheless, evidence of some modification of the course of infections was obtained in some, but not in all, cases.

2. Splenectomized S.sinicus infected with P.knowlesi.

(a). Untreated.

Removal of the spleen from sinicus monkeys renders them more susceptible to the pathogenic effects of P.knowlesi than intact sinicus monkeys. As a rule, the course of P.knowlesi infections in splenectomized sinicus monkeys is rapidly progressive and ultimately fatal. In a small proportion of cases, however, spontaneous recovery takes place; and it may be assumed, therefore, that infections with P.knowlesi in splenectomized sinicus monkeys are, if anything, slightly less severe than similar infections in non-splenectomized rhesus monkeys. Since all the splenectomized sinicus monkeys which received serum treatment died in the acute attack, further reference to controls is omitted.

(b). Treated.

Five sinicus monkeys which were splenectomized and subsequently infected with P.knowlesi were treated with immune serum. Details of these experiments are given in Table II. In every instance, death occurred within 4 to 6 days of the appearance of parasites in the peripheral blood, and it is apparent, therefore, that the administration of immune serum was of no value in modifying the course of the infection in any of these monkeys.

3. S.rhesus infected with P.knowlesi.

(a). Untreated.

P.knowlesi causes a rapidly progressive

and almost invariably fatal, infection in S.rhesus. In a series of over 120 rhesus monkeys infected with P.knowlesi, Mulligan and Sinton (1933) observed only one case of spontaneous recovery. Subsequent experience has shown that, in spite of repeated passage of the same strain by blood inoculation, the pathogenicity of P.knowlesi for Rhesus monkeys has not diminished. Coggeshall and Kumm (1937) observed only one instance of spontaeous recovery in a series of over 70 rhesus monkeys infected with P.knowlesi.

(b). Treated.

Details of five normal intact rhesus monkeys infected with P.knowlesi and subsequently treated with superinfected serum, are given in Table III. Serum treatment was commenced as soon as parasites were detected in the peripheral blood. All of the five animals died, but there was evidence in at least two cases that the course of the primary attack was modified as the result of treatment. Monkeys 71 and 75 died on the 10th and 12th days, respectively, after parasites were detected in the peripheral blood. Monkey 75 received quinine treatment on the 10th and 11th days of the infection. Monkeys 82 and 113 died on the 7th day of infection, and monkey 93 on the 5th day. It may be concluded, therefore, that serum treatment was of some slight value in certain cases.

4. Stimulated S.rhesus infected with P.knowlesi.

The partial success of immune serum therapy in modifying the course of knowlesi infections in intact sinicus and rhesus monkeys, and its complete failure in splenectomized sinicus monkeys infected with P.knowlesi, suggested that the lymphoid-macrophage system might be an important intermediary in the utilization of protective antibodies. It was decided,

therefore, to observe the effects of immune serum therapy in monkeys in which the lymphoid-macrophage system had been "stimulated" prior to infection with P.knowlesi. For this purpose, rhesus monkeys with chronic, or latent, infections with P.cynomolgi were employed. The latter infection causes considerable hyperplasia of the lymphoid-macrophage systems in most cases, the degree of stimulation being in direct proportion to the severity of the primary attack. Stimulation of the lymphoid-macrophage system per se is ineffective in controlling infection with a heterologous species, or strain, of Plasmodium (Taliaferro and Mulligan, loc.cit.)

(a). Untreated.

Rhesus monkeys suffering from chronic or latent infections with P.cynomolgi are as susceptible to the pathogenic effects of P.knowlesi as previously normal monkeys.

(b). Treated.

Ten rhesus monkeys were infected with P.cynomolgi, and the infections were allowed to run their natural course. In eight of these ten monkeys, the primary attack with P.cynomolgi was well developed and in the remaining two (106 and 111), only mild transient infections resulted. At intervals varying from 39 to 232 days after first infection, these monkeys were cross-infected with P.knowlesi and, as soon as the latter parasite was detected in the peripheral blood, treatment with serum obtained from monkeys with chronic or latent knowlesi infections was instituted. Details of these experiments are given in Table IV.

Although the daily dose of immune serum administered to individual monkeys in this series

was, in most cases, less than that given to unstimulated rhesus monkeys (Table III), or splenectomized sinicus monkeys (Table II), the efficacy of the serum in controlling knowlesi infections was very much more pronounced in most cases. Seven of the ten monkeys in this group (729, 90, 91, 92, 96, 97 and G2) developed mild infections, none of which exceeded 10 per cent of parasitized red cells on the day of maximum parasite prevalence. There was no reason to believe that the sera employed in the treatment of these seven monkeys were richer in antibodies than those used in the treatment of unstimulated or splenectomized monkeys. Monkey 110 developed a moderately severe attack, but the beneficial effects of serum treatment were indicated by the slow rate of increase of the parasites, with a consequent prolongation of the primary attack. Unfortunately, owing to lack of a sufficient quantity of immune serum, treatment had to be discontinued on the 9th day, and this monkey succumbed on the 12th day. The two remaining monkeys in this series succumbed to the infection on the 8th day after P.knowlesi had been detected in the peripheral blood. It appears to be significant that the two monkeys in this series (106 and 111), which derived least benefit from treatment with immune serum, were the two animals which were most resistant to infection with P.cynomolgi. It seems probable that the mild transient infection with P.cynomolgi which occurred in these two monkeys, failed to produce an adequate degree of stimulation of the lymphoid-macrophage system. This view is supported by the observation that no appreciable degree of lymphoid-macrophage stimulation has been observed in sinicus

monkeys which have recovered from mild transient infections with P.cynomolgi. It is possible, however, that the inefficacy of serum treatment in these animals may have been due, in part at least, to the use of less potent serum.

The results of these experiments suggest that the efficacy of homologous immune serum in controlling knowlesi infection in rhesus monkeys is directly correlated with the degree of stimulation of the lymphoid-macrophage system in animals receiving treatment.

5. S.sinicus infected with P.cynomolgi.

P.cynomolgi produces only a very mild and transient infection in sinicus monkeys. In a series of ten monkeys, in which the course of the infection was followed by blood films taken at four-hourly intervals, it was found that, at the height of parasite prevalence in any individual monkey, not more than 1.5 per cent of the erythrocytes were parasitized. It was thought that it might be possible to reinforce this high degree of natural immunity by the administration of immune serum, and thereby prevent the occurrence of detectable infections. Four sinicus monkeys were inoculated with P.cynomolgi, but, in spite of the administration of homologous serum from superinfected sinicus monkeys, all of them developed infections which did not appear to be of less severity than the controls.

RESULTS OF EXPERIMENTS WITH SPLEEN EXTRACTS.

6. S.sinicus infected with P.knowlesi.

(a). Untreated.

The results of infection with P.knowlesi in 25 untreated sinicus monkeys have been described.

Individual monkeys varied considerably in their susceptibility to P.knowlesi, and the infections varied from relatively mild ones (5 per cent red cells parasitized at peak of infection) to sever and sometimes fatal infections (over 70 per cent parasitized red cells at peak of infection). In most cases, attacks of moderate severity occurred from which recovery was spontaneous, but some monkeys (28 per cent) developed severe attacks and died.

(b). Treated.

Nine sinicus monkeys were treated with spleen extract after infection with P.knowlesi. The results of these experiments are given in Table V. Owing to the wide variation in the intensity of knowlesi infections in untreated monkeys, it was difficult to assess the value of treatment with spleen extracts. Two of the nine treated monkeys, (59, 67) developed severe fatal infections, and it may be assumed that neither of them derived any appreciable benefit from treatment. Four others (20, 42, 43, 52) developed infections of moderate severity, such as are commonly seen in untreated monkeys. The three remaining monkeys (24, 31, 60) developed very mild transient infections, which were of less intensity and shorter duration than any of those in the control group. One of these three monkeys (31) was treated with extract of spleen obtained from a sinicus monkey with an acute infection, another (24) with spleen extract from a sinicus monkey with a chronic infection, and the third (60) with spleen extract obtained from a superinfected sinicus monkey.

The results of treatment with spleen extracts in sinicus monkeys infected with P.knowlesi were similar to those observed in sinicus monkeys treated

with homologous serum. It cannot be claimed that any very definite or constant benefit was derived from treatment with spleen extract, but it seems probable that the course of the initial infection was modified to some extent in at least three cases.

7. Splenectomized S.sinicus infected with P.knowlesi.

(a). Untreated.

Splenectomized sinicus monkeys were found to be highly susceptible to P.knowlesi, and rapidly progressive attacks ending fatally within a week were the rule. Such monkeys were, however, less susceptible to P.knowlesi than intact rhesus monkeys, as evidenced by the fact that a small proportion recovered spontaneously. All the splenectomized monkeys in the treated group died, and further reference to controls, is, therefore, unneccessary.

(b). Treated.

Six splenectomized sinicus monkeys infected with P.knowlesi were treated with homologous spleen extracts (Table VI). All developed acute attacks and died. Two of these six monkeys died suddenly, after the 6th and 7th injections of spleen extract respectively. As all of the six monkeys showed a high degree of parasitæmia before death occurred, it may safely be concluded that the administration of spleen extract was of no value. Similar results were obtained in splenectomized sinicus monkeys treated with immune serum.

8. Rhesus infected with P.knowlesi.

(a). Untreated.

The course of P.knowlesi infections in S.rhesus has been described. For practical purposes, it may be accepted that, in untreated rhesus monkeys

P.knowlesi causes a rapidly progressive infection, which proves fatal in from 4 to 6 days after the appearance of parasites in the peripheral blood.

(b). Treated.

Five rhesus monkeys infected with P.knowlesi were treated with homologous spleen extracts, the injections being commenced as soon as parasites were detected in the peripheral blood (Table VII). All died with heavy parasitic infections. In one case (monkey 68), death did not occur until the 9th day after parasites were detected, and the rate of increase in the number of parasites was less rapid than in untreated animals. It is probable, therefore, that some slight benefit was derived from the administration of spleen extract in this one instance. These results correspond closely with those obtained in rhesus monkeys treated with immune serum.

9. Stimulated S.rhesus infected with P.knowlesi.

(a). Untreated.

As has been pointed out, rhesus monkeys, in which the lymphoid-macrophage system has been "stimulated" by previous infection with P.cynomolgi, are as susceptible to the pathogenic effects of P.knowlesi as previously uninfected monkeys. Infections with P.knowlesi in adequately stimulated monkeys were readily controlled by the administration of homologous immune serum.

(b). Treated.

Six rhesus monkeys, in which adequate lymphoid-macrophage stimulation was believed to have been produced by previous infection with P.cynomolgi were infected with P.knowlesi, and treated with

homologous spleen extracts. Detils of these experiments are given in Table VIII. Monkeys 89 and 120 did not appear to have benefited from the administration of spleen extract and died with severe infections on the 5th and 6th days respectively after parasites appeared in the peripheral blood. The remaining four monkeys in this group appeared to have derived some benefit from the administration of spleen extract, as evidenced by the slower rate of increase in parasite prevalence. All four monkeys showed evidence of toxicity as the result of the injections, and it is possible that the beneficial results of treatment may thereby have been offset to some extent. Monkeys 121, 122 and 123 showed some disinclination for food shortly after the treatment was commenced, and all of them became listless and anaemic before parasites were sufficiently prevalent to account for these symptoms. Injections of spleen extract were withheld if there was no tendency for the parasite count to rise rapidly. Monkey 616 is of special interest inasmuch as it was first infected with P.cynomolgi about 4 years before being infected with P.knowlesi. During this period, infection with P.cynomolgi had been eradicated by natural means and, after re-infection, the infection had been eradicated by intensive drug therapy before the monkey was re-infected for the second time. A small piece of spleen was removed from this monkey at biopsy shortly before it was infected with P.knowlesi, sections of which showed a high degree of lymphoid-macrophage stimulation. As soon as P.knowlesi appeared in the peripheral blood, treatment with homologous spleen extract was commenced.

On the 5th day after P.knowlesi appeared in the peripheral blood, only 6 per cent of the red cells were parasitized. Unfortunately, the monkey died on the following day. Death was probably caused by the toxic effects of the relatively large doses of spleen extract administered.

Although the number of monkeys in this group is small, the results obtained suggest that the administration of spleen extracts was of more value than in similar experiments in splenectomized or non-stimulated monkeys. These findings correspond closely with those obtained in the experiments with immune sera reported previously.

10. S.sinicus infected with P.cynomolgi.

(a). Untreated.

P.cynomolgi produces only a very mild infection in sinicus monkeys. In a series of ten such monkeys, the course of infection with P.cynomolgi, was carefully studied by taking blood films at four-hourly intervals. In none of these animals did the degree of parasitaemia at the peak of infection exceed 1.5 per cent of parasitized red cells.

(b). Treated.

Five sinicus monkeys were infected with P.cynomolgi, and treated with extracts of spleens of sinicus monkeys, which had been infected and super-infected with the homologous strain of Plasmodium. Four of these five monkeys developed attacks which did not appear to be of less severity than those seen in the control. The remaining monkey showed a very scanty and transient infection.

CONCLUSIONS.

No evidence was obtained that the course of P.cynomolgi infections in sinicus monkeys was, in

any way, modified by the administration of either serum obtained from sinicus monkeys with chronic or latent infections caused by the homologous species, or with spleen extracts prepared from the spleen of monkeys infected with the same plasmodium.

The administration of large doses of homologous immune serum appeared to modify to a slight extent the course of knowlesi infections in intact sinicus and rhesus monkeys, but had no effect on the course of similar infections in splenectomised sinicus monkeys. The effect of homologous immune serum in controlling knowlesi infections in rhesus monkeys, which had previously suffered from an acute attack of malaria caused by P.cynomolgi, was clearly demonstrated.

Evidence was obtained that the administration of homologous spleen extracts modified the course of infection with P.knowlesi in some intact sinicus and rhesus monkeys. The influence of spleen extracts on the course of knowlesi infections was greatest in rhesus monkeys, which had previously suffered from an acute attack of malaria caused by P.cynomolgi.

Spleen extracts in the doses employed in these experiments, were of less value in controlling malarial infections in monkeys than immune sera. No evidence of any great concentration of protective antibodies in the spleen was obtained.

The chief interest of the experiments reported in this section lies in the results obtained with immune serum and spleen extracts in controlling the course of P.knowlesi infections in monkeys, which had previously suffered from an acute attack of malaria caused by P.cynomolgi. It would seem from

these results that the mechanism of defence against malaria involves the interaction of both cellular and humoral agencies. The effective control of the disease is only possible when there is a full measure of one of these factors operating in the presence of an adequate measure of the other. It might be argued that a cross immunity exists between infections with P.cynomolgi and those with P.knowlesi, but this is not borne out by experience. A more reasonable interpretation of the result would appear to be that the utilisation of the protective antibodies was facilitated by the presence of a sufficient degree of stimulation of the cells of the lymphoid-macrophage system, brought about by previous infection with P.cynomolgi. An alternative explanation would be that the stimulated cells of the lymphoid-macrophage system are relatively impotent in the absence of the specific protective antibody.

At present it seems to me, in the absence of detailed evidence on the parts played by cells and antibodies in malarial defence, discussion on the relative importance of the one compared to the other is as futile as an argument on whether Britain or U.S.A. won the war. The fact remains that for the successful control of a malarial infection in the host both agencies must be operative.

TABLE I.

Protocols of 18 normal intact S.sinicus monkeys infected with P.knowlesi and treated with homologous serum.

S.sinicus number.	P.knowlesi parasites injected in millions.	Donor of Serum £		Dosage of serum in c.cm. Percentage of parasitized red cells. on days after inoculation with P.knowlesi.															
		Species and number.	Stage of infection.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
19	500	S.rhesus 473	Acute	-	-	-	-	-	-	15	10	-	10	10	10	10	-	-	-
				-	-	-	P	1	4	7	7	6	8	5	4	1	P	P	P
30	500	S.sinicus 23	"	-	-	5	5	5	5	5	5	7	-	-	-	-	-	-	-
				-	-	P	1	3	4	4	3	5	3	1	P	P	P	-	-
66	5	" 64	"	-	-	-	-	5	10	10	10	5	7	-	-	-	-	-	-
				-	-	-	P	1	4	11	11	13	28D	-	-	-	-	-	-
77	5	" 45	Acute (splenectomy).	5	5	5	5	5	5	5	5	5	5	5	5	-	-	-	-
				-	-	-	-	P	2	6	12	10	10	7	4	3	1	P	P
78	5	S.rhesus 421	Acute	5	5	5	5	5	5	5	5	5	5	5	5	-	-	-	-
				-	-	-	-	P	1	3	9	10	10	9	7	7	5	DO	-
32	500	S.sinicus 28	Sub-acute	5	5	5	5	5	5	5	5	-	-	-	-	-	-	-	-
				-	-	-	-	P	2	1	3	1	1	P	P	P	P	-	-
33	500	" 28	"	5	5	5	5	5	5	5	5	-	-	-	-	-	-	-	-
				-	-	-	P	1	5	8	16	5	8	7	3	2	P	P	-
34	500	" 28	"	-	-	-	5	5	5	5	5	-	-	-	-	-	-	-	-
				-	-	-	P	P	2	9	5	4	4	2	2	P	P	-	-
35	500	" 28	"	-	-	-	5	5	5	5	5	-	-	-	-	-	-	-	-
				-	-	-	P	1	3	6	5	5	2	3	1	P	P	-	-
36	500	" 28	"	-	-	-	-	5	5	5	5	5	5	-	-	-	-	-	-
				-	-	-	-	P	4	6	7	8	4	9	3	1	P	-	-
37	500	" 28	"	-	-	-	-	-	-	-	10	10	5	5	-	-	-	-	-
				-	-	-	-	P	P	3	7	10	8	4	4	2	P	P	-
38	500	" 28	"	-	-	-	-	-	-	10	10	5	5	-	-	-	-	-	-
				-	-	-	P	2	7	12	18	17	22D	-	-	-	-	-	-
39	500	" 28	"	-	-	-	-	-	-	10	10	5	5	-	-	-	-	-	-
				-	-	-	-	P	3	6	12	5	3	4	1	P	-	-	-
21	100	" 20	Chronic	-	-	-	-	-	10	10	13	-	-	-	-	-	-	-	-
				-	-	-	-	1	10	26	29	7	4	3	P	P	-	-	-
25	10	" 19 &20	"	-	-	2	2	2	2	6	6	6	-	-	-	-	-	-	-
				-	-	P	1	6	5	2	1	2	2	3	1	P	P	-	-
51	5	" 33	Superinfected	5	5	5	5	5	5	-	-	-	5	5	5	-	-	-	-
				-	-	-	-	-	P	P	1	1	P	P	P	P	-	-	-
55	5	" 39	"	-	-	-	-	-	5	5	5	5	5	5	5	5	-	-	-
				-	-	-	-	1	5	12	14	12	8	11	13	10	5	1	-
62	100	" 32 &37	"	-	10	10	10	10	10	10	5	-	-	-	-	-	-	-	-
				-	P	P	2	2	4	5	3	8	5	4	5	3	2	1	P

£ vide Appendix
D = died or sacrificed when moribund.
P = less than 1 per cent of red cells parasitized.
DO = died from causes other than malaria.

TABLE II.

Protocols of five splenectomized S.sinicus monkeys infected with P.knowlesi and treated with homologous serum.

S.sinicus number.	P.knowlesi parasites injected in millions.	Donor of Serum £		Dosage of serum in c.cm. on days after inoculation with P.knowlesi. Percentage of parasitized red cells.									
		Species and number.	Stage of infection.	1	2	3	4	5	6	7	8	9	10
1*	1,000	S.sinicus 62	Acute	-	-	10	10	10	-	-	-	-	-
				-	-	2	11	60	65D	-	-	-	-
2*	1,000	S.rhesus 72	"	-	-	-	-	-	10	10	10	10	-
				-	-	-	-	-	1	2	14	33D	-
16*	5	S.sinicus 32	Superinfected	-	5	5	5	5	5	5	5	5	-
				-	-	-	-	P	3	34	35	56D	-
18*	5	" 37	"	-	-	-	5	5	5	5	5	5	-
				-	-	-	1	4	18	29	48	54D	-
57	5	" 35	"	-	5	5	5	5	-	-	-	-	-
				-	P	P	4	32	54D	-	-	-	-

* Previously infected with P.cynomolgi.
£ vide Appendix.
D = died or sacrificed when moribund.
P = less than 1 per cent of red cells parasitized.

TABLE III.

Protocols of five S.rhesus monkeys infected with P.knowlesi and treated with homologous serum.

S.rhesus number.	P.knowlesi parasites injected in millions.	Donor of Serum £		Dosage of serum in c.cm. on days after inoculation with P.knowlesi.											
		Species and number.	Stage of infection.	Percentage of parasitized red cells.											
				1	2	3	4	5	6	7	8	9	10	11	12
71	5	S.sinicus 29	Superinfected	5	5	5	5	12	10	5	5	5	5	-	-
				P	1	4	7	10	9	13	22	36	49D	-	-
75	5	" 31	"	5	5	5	5	5	5	12	10	5	5Q	Q	-
				P	P	1	3	3	7	16	23	42	41	16	5D
82	5	" 44	"	5	10	10	10	10	10	10	-	-	-	-	-
				P	P	P	2	9	40	77D	-	-	-	-	-
93	5	S.rhesus 720	"	7.5	7.5	10	10	-	-	-	-	-	-	-	-
				P	4	17	35	54D	-	-	-	-	-	-	-
113	5	" 720	"	7.5	7.5	7.5	7.5	7.5	-	-	-	-	-	-	-
				P	1	2	8	17	32	90D	-	-	-	-	-

£ vide Appendix
D = died or sacrificed when moribund.
P = less than 1 per cent of red cells parasitized.
Q = quinine treatment.

TABLE IV.

Protocols of ten "stimulated" S.rhesus monkeys infected with P.knowlesi and treated with homologous serum.

S.rhesus number.	Duration of P.cynomolgi infection in days.	P.knowlesi parasites injected in millions.	Donor of Serum £		Dosage of serum in c.cm. on days after P.knowlesi detected Percentage of parasitized red cells in peripheral blood.																	
			Species and number	Stage of infection	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
729	232	2	S.sinicus 34, 35.	Super-infected.	5 P	5 P	5 P	5 P	5 P	5 P	5 P	- P	- P	-	-	-	-	-	-	-	-	-	-	-
90	40	10	S.rhesus 705	"	10 P	10 P	10 2	10 5	10 5	10 6	10 7	10 5	10 4	- 2	- 2	- 2	- 2	- 2	- 2	- P	- P	- P	- P	- P
91	48	5	S.sinicus 51, 56, 60	"	5 P	10 2	5 2	5 5	5 5	5 4	5 3	5 2	5 1	- 2	- 1	- 1	- 1	- 1	- 1	- P	- P	- P	- P	- P
92	75	5	"	"	5 P	5 P	5 P	5 2	5 3	5 2	5 3	5 2	5 2	5 2	- 2	- 4	- 3	- 2	- 2	- 3	- 1	- P	- P	- P
96	39	5	S.rhesus 731, 732.	"	5 P	5 P	5 P	5 P	5 P	5 2	5 2	5 5	7.5 4	7.5 4	5 3	5 2	- 1	- 3	- 3	- 2	- P	- P	- P	- P
97	39	5	"	"	5 P	5 P	5 P	5 P	5 P	- P	- P	- P	5 1	5 2	5 2	- 2	- 1	- 1	5 3	- 2	- 2	- 3	- 1	- P
G2	85	100	S.rhesus 91, 92.	"	5 P	5 3	7.5 4	7.5 6	7.5 8	7.5 7	7.5 6	7.5 8	7.5 9	- 7	- 4	- 1	- 1	- P	- P	- P	- P	- P	- P	- P
106	63	5	S.sinicus 107,108,109.	"	5 1	5 1	5 4	5 8	5 21	10 24	10 27	- 57D	-	-	-	-	-	-	-	-	-	-	-	-
110	44	5	"	"	5 P	5 P	5 1	5 2	5 6	10 8	5 10	5 19	7.5 23	- 26	- 29	- 31D	-	-	-	-	-	-	-	-
111	44	5	S.rhesus 96	Chronic	5 P	5 1	5 4	5 6	5 21	10 27	10 29	- 43D	-	-	-	-	-	-	-	-	-	-	-	-

£ vide Appendix.
D = died or sacrificed when moribund.
P = less than 1 per cent or red cells parasitized.

TABLE V.

Protocols of nine S.sinicus infected with P.knowlesi and treated with homologous spleen extract.

S.sinicus number.	Donor of Spleen: Species	Donor of Spleen: Stage of infection.	Weight of spleen substance extracted in grammes	Dosage of spleen extract in c.cm. / Percentage of parasitized red cells. on days after inoculation with P.knowlesi.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
31	S.sinicus	Acute	7		-	-	5	5	5	5	5	-	-	-	-	-	-	-	-	-	-	-	-	-
					-	-	P	2	3	2	1	1	P	P	P	P	-	-	-	-	-	-	-	-
67	"	"	12		-	-	-	-	-	5	10	5	10	10	10	-	-	-	-	-	-	-	-	-
					-	-	-	-	-	P	4	8	11	13	12	28D	-	-	-	-	-	-	-	-
42	"	Sub-acute	22		-	-	-	5	5	5	5	5	-	-	-	-	-	-	-	-	-	-	-	-
					-	-	-	P	P	5	6	6	5	2	1	P	P	P	P	-	-	-	-	-
43	"	"	22		-	-	-	5	5	5	5	5	-	-	-	-	-	-	-	-	-	-	-	-
					-	-	-	P	2	4	6	6	3	1	1	P	P	P	P	-	-	-	-	-
20	"	Chronic	22		10	10	10	10	10	10	10	Q	Q	-	-	-	-	-	-	-	-	-	-	-
					-	-	P	2	3	4	13	6	2	P	P	P	P	-	-	-	-	-	-	-
24	"	"	5		-	-	-	-	10	10	6	-	-	-	-	-	-	-	-	-	-	-	-	-
					-	-	-	-	P	P	3	3	3	2	1	1	P	P	P	P	-	-	-	-
52	"	Superinfected	6		-	5	5	5	5	5	5	-	-	-	-	-	-	-	-	-	-	-	-	-
					-	-	-	-	-	P	P	P	7	3	4	1	1	3	1	P	P	P	P	-
59	"	"	18		10	10	10	10	10	10	10	10	-	-	-	-	-	-	-	-	-	-	-	-
					-	P	1	1	5	6	10	21D	-	-	-	-	-	-	-	-	-	-	-	-
60	"	"	8		5	5	5	5	5	5	4	-	-	-	-	-	-	-	-	-	-	-	-	-
					-	P	P	P	2	3	4	3	5	2	2	2	2	1	2	2	1	1	P	P

Q = quinine treatment.
D = died.
P = less than 1 per cent of red cells parasitized.

TABLE VI.

Protocols of six splenectomized S.sinicus infected with P.knowlesi and treated with homologous spleen extract.

S.sinicus number.	Donor of Spleen: Species	Donor of Spleen: Stage of infection.	Weight of spleen substance extracted in grammes	Dosage of spleen extract in c.cm. Percentage of parasitized red cells on days after inoculation with P.knowlesi.															
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
7	S.sinicus	Sub-acute	10	–	–	10	10	10	10	–	–	–	–	–	–	–	–	–	–
				–	–	P	2	19	38	58D	–	–	–	–	–	–	–	–	–
10	"	Superinfected	7	–	–	–	–	–	–	–	–	–	–	10	10	10	5	–	–
				–	–	–	–	–	–	–	–	–	–	1	3	9	46D	–	–
27	"	"	8	5	5	5	5	5	5	–	–	5	–	–	–	–	–	–	–
				–	–	–	–	–	–	P	4	48D	–	–	–	–	–	–	–
49	"	"	11	–	–	–	–	–	5	5	5	5	5	5	3	3	6	Q	Q
				–	–	–	–	–	P	P	2	3	2	5	6	20	41	36	19D
50	"	"	7	–	–	–	–	5	5	5	5	5	–	–	–	–	–	–	–
				–	–	–	–	P	P	6	16	27	31D	–	–	–	–	–	–
58	"	"	6	–	–	–	–	5	5	5	5	5	–	–	–	–	–	–	–
				–	–	–	–	P	1	9	28	60D	–	–	–	–	–	–	–

Q = quinine treatment
D = died.
P = less than 1 per cent of red cells parasitized.

TABLE VII.

Protocols of five S.rhesus infected with P.knowlesi and treated with homologous spleen extract.

S.rhesus number.	Donor of Spleen: Species	Donor of Spleen: Stage of infection.	Weight of spleen substance extracted in grammes	Dosage of spleen extract in c.cm. / Percentage of parasitized red cells. on days after inoculation with P.knowlesi.															
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
68	S.sinicus	Superinfected	9	-	-	-	-	-	-	5	5	5	5	5	5	10	5	-	-
				-	-	-	-	-	-	P	P	1	6	12	21	27	40	50D	-
73	"	"	8	-	-	-	-	-	-	-	5	5	10	10	5	-	-	-	-
				-	-	-	-	-	-	-	P	6	17	56	100D	-	-	-	-
74	"	"	7	-	-	-	-	-	5	5	5	5	12	10	5	-	-	-	-
				-	-	-	-	-	P	P	1	7	31	51	48D	-	-	-	-
81	"	"	8	-	-	-	-	-	-	-	10	10	10	10	10	-	-	-	-
				-	-	-	-	-	-	-	P	1	4	33	46D	-	-	-	-
83	"	"	13	-	-	-	-	10	10	10	10	10	10	-	-	-	-	-	-
				-	-	-	-	P	P	P	3	19	45	80D	-	-	-	-	-

D = died.

P - less than 1 per cent of red cells parasitized.

TABLE VIII.

Protocols of six "stimulated" S.rhesus infected with P.knowlesi and treated with homologous spleen extract.

S.rhesus number.	Days after first infection with P.cynomolgi	Donor of Spleen: Species	Donor of Spleen: Stage of infection	Weight of spleen substance extracted in grammes.	Dosage of spleen extract in c.cm. on days after inoculation with P.knowlesi. Percentage of parasitized red cells.																				
					1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
89	38	S.sinicus	Super-infected	6	-	-	-	-	-	5	10	10	10	-	-	-	-	-	-	-	-	-	-	-	-
					-	-	-	-	-	P	9	13	41	D	-	-	-	-	-	-	-	-	-	-	-
120	31	"	"	8	-	-	-	-	10	10	10	5	5	5	-	-	-	-	-	-	-	-	-	-	-
					-	-	-	-	P	1	5	19	30	41	D	-	-	-	-	-	-	-	-	-	-
121	31	"	"	8	-	-	-	-	10	10	10	5	5	5	-	-	-	-	-	-	-	-	-	-	-
					-	-	-	-	P	1	4	11	22	24	33	D	-	-	-	-	-	-	-	-	-
122	31	S.sinicus		17	-	-	-	10	10	10	5	5	-	-	-	-	5	5	5	-	5	-	-	-	-
					-	-	-	-	-	-	-	-	P	P	P	P	2	4	6	8	20	D	-	-	-	-
123	31	S.rhesus	"	22	-	-	-	10	10	10	5	5	-	5	5	-	5	5	5	-	5	-	-	-	-
					-	-	-	P	P	P	P	P	2	5	6	4	10	16	16	16	17	14	14	11	D
616	1,584	S.rhesus	"	17	-	-	-	-	-	-	10	10	10	10	10	-	-	-	-	-	-	-	-	-	-
					-	-	-	-	-	-	P	P	P	1	6	D	-	-	-	-	-	-	-	-	-

D = died.

P = less than 1 per cent of red cells parasitized.

APPENDIX.

Summary of histories of S.sinicus and S.rhesus monkeys infected with P.knowlesi from which "immune" serum was obtained.

Monkey species and number.	Percentage of parasitized red cells on day of maximum parasite prevalence	Days after first infection with P.knowlesi when serum obtained.	Stage of infection at which serum obtained. £	Treatment, if any, given during primary attack.
S.sinicus 19	8	128	Chronic	Serum
" 20	13	13	"	Quinine
" 23	24	14	Acute	Nil
" 28	19	10	Sub-acute	"
" 29	10	145	Superinfected	"
" 31	3	145	"	Spleen Extract
" 32	3	26	"	Serum
" 33	16	26	"	"
" 34	9	26	"	"
" 35	6	26	"	"
" 37	10	26	"	"
" 39	12	26	"	"
" 44	20	144	"	Nil
" 45	40	98	Acute following splenectomy	"
" 51	1	219	Superinfected	Serum
" 56	20	127	"	Nil
" 60	4	176	"	Spleen Extract
" 62	8	48	Acute following splenectomy	Serum
" 64	68	10	Acute	Nil
S.rhesus 72	72	7	"	
" 91*	5	71	Superinfected	Serum
" 92*	4	71	"	"
" 96*	5	71	Chronic	"
S.sinicus 107	13	67	Superinfected	Nil
" 108	16	67	"	"
" 109	21	67	"	Quinine
S.rhesus 421*	Primary attacks treated with quinine. No parasite counts available.	10	Acute	"
" 473		16	"	"
" 705		Over one year	Superinfected	"
" 720		"	"	"
" 731		148	"	"
" 732		148	"	"

SECTION VII.

MALARIA IN THE MALABAR SQUIRREL.

INTRODUCTION.

The reading of a report by Donovan (1920) of a natural malarial infection in the Malabar squirrel aroused interest in this infection, as it was felt that it would greatly facilitate experimental studies on malaria if it could be transmitted to one of the small mammals commonly used in the laboratory. It was decided, therefore, to investigate the problem.

The scientific name for the Malabar squirrel is given as Sciurus indicus malabaricus (Blandford, 1888), but it is variously recorded as Sciurus malabaricus, Sciurus maximus and Ratufa indica malabarica. The head and body of the adult squirrel measures about 18 inches and the bushy tail is of approximately the same length. It is a beautiful creature to look at, being a deep maroon colour on the dorsal aspects of the head, back, rump and the whole of the tail, while the ventral surfaces are yellowish brown or buff. These animals abound in the dense forests of the Nilgiri-Wynaad where they inhabit tall trees, living among the branches and rarely coming to the ground. The young are reared in nests built of twigs and leaves near the top of lofty trees.

In order to obtain specimens of the Malabar squirrel it was necessary to organise "a shoot" and permission had to be obtained from the Nilgiri's Game Association because of the strict regulations which were then in force for its preservation. Six squirrels were shot during an expedition to the Game Reserve near Tappikadu, on the main road between Ootacamund and Mysora City, that is, in the same locality as that in which Donovan had

had made his observations twenty years earlier. Later three squirrels were captured alive. Seven out of the nine squirrels examined showed the presence of a malaria parasite in their blood.

Description of the Plasmodium.

The same species of plasmodium was present in all of the seven squirrels found to be infected. In every case gametocytes were prevalent but asexual forms were always scanty. The description given below is based on the examination of blood films stained by Giemsa's method. As I am no artist and as there were no facilities for microphotography I have decided to present the observations in the form of a word description.

Asexual Cycle.

Trophozoites were invariably scanty and true schizogony was not observed either in the peripheral blood or in the internal organs. The prevalence of gametocytes makes it probable that at least some of the developing forms to be described were immature sexual forms. No characteristic changes were observed in the size, shape or staining reactions of the infected red cells at any stage of development of the parasite.

The small trophozoites seen consisted of a single, round chromatin dot and a semi-lunar or oval wisp or blob of blue-staining protoplasm. Small signet-ring forms were not observed but larger rings measuring 1/2 to 2/3 the diameter of the red cell were occasionally seen, in these, the chromatin nucleus occurred as a single mass placed opposite to the thickened portion of the protoplasmic ring; in the larger forms a few discrete granules of dark-brown pigment were seen. Forms showing little or no vacuolation but which otherwise

appeared to be at a similar stage of development probably represented developing gametocytes. Amœboid forms presented a variety of appearances, the protoplasm was very irregular and appeared to be fragmented; the chromatin nucleus occurred as a single mass or as several fragments; a few small dark-brown pigment granules were sometimes seen. Larger forms were more regular in outline and contained more numerous and somewhat coarser pigment granules; as a rule there was considerable vacuolation and when this was single the parasite appeared as a large ring occupying the greater part of the red cell; the chromatin nucleus usually occurred as a single mass which was more or less dense and compact, or consisted of an aggregation of chromatin granules. Although trophozoites measuring 5/u to 6/u in diameter were frequently observed, forms showing true schizogony were not seen.

Sexual Cycle.

Mature gametocytes, which were plentiful in most of the blood films examined, occurred as more or less spherical bodies free from all traces of the original host red cell. The sexes were readily distinguishable, and females were rather more numerous than males; a differential count made on one blood film showed the male : female ratio to be 4 : 5.

Macrogametocytes were slightly larger than a normal red cell and commonly measured about 8/u in diameter. The protoplasm stained deep blue and usually presented a somewhat granular appearance; as a rule the protoplasm was seen as a more or less solid, but not infrequently it was vacuolated. The chromatin

nucleus was characterized by its relatively small size, its granular appearance and vivid red staining; the nucleus was situated centrally or peripherally and was composed of a single mass, or of two or even three small masses lying close together; the shape of the nucleus was very variable being round, oval, linear or irregular; occasionally the nucleus consisted of one or two small dense compact masses. The pigment granules were dark-brown in colour and were more or less evenly scattered through the protoplasm which, by reason of its deep staining, tended to obscure them.

Microgametocytes were readily distinguished by the faint staining of the protoplasm, the large size of the nucleus and the clarity with which the pigment granules stood out. There was greater disparity in size than in the case of the macrogametocytes and forms varying in size from 6 μ to 8 μ in diameter were commonly seen. The nucleus which was composed of an outer faintly staining zone and an inner deeply staining zone (karyosome) occupied a considerable proportion of the parasite. The karyosome was larger and more homogeneous than the nucleus of the macrogametocyte and often stained a deep dull red or purple colour; the outer zone of the nucleus which stained a pinkish colour tended to merge almost imperceptibly into the protoplasm. The protoplasm which was seldom vacuolated stained a faint bluish or greenish-grey colour against which the dark-brown pigment granules were very clearly seen. The pigment granules were more or less evenly scattered through the protoplasm but tended to be coarser an more plentiful towards the periphery; the size of the granules was variable in different parasites.

Duration of Schizogony Cycle.

It has not been possible to work out the duration of the schizogony cycle on the material available.

Exo-erythrocytic Development.

A detailed search of sections of spleen, liver, kidney, lung and intestine of infected squirrels revealed no evidence of an exo-erythrocytic development of this plasmodium.

Pathogenicity.

The apparent healthiness of the animals in their natural habitat in spite of a high incidence of infection suggests that the disease in the adults is of low pathogenicity. This is supported by the observations on two infected adults which were maintained in captivity for many months,as they remained in good health.

Infectivity for Other Animals.

As the primary object of this investigation was to establish an experimental malarial infection in a small laboratory animal, attemps to transmit the squirrel malaria received special attention. Blood obtained from all of the infected squirrels was inoculated chiefly into guinea-pigs, rabbits and white rats. On a few occasions attempts were also made to transmit the infection by blood inoculation to monkeys (S.sinicus). In the case of white rats and monkeys both intact and splenectomized animals were used.

The blood used for inoculation was obtained both from the squirrels shot in the jungle and from the two infected squirrels which were maintained in captivity. In the former case the thorax was opened immediately the animals fell and 10cc. to 20cc. of blood were withdrawn into sterile syringes containing citrate-saline

solution as an anticoagulant. In every case bleeding was effected before the heart stopped beating. The citrated blood was transported to the laboratory in a portable ice box and was used for the subinoculation experiments within 4 to 8 hours of removal from the donor. Cardiac puncture of the infected captive squirrels was carried out on several occasions without difficulty or mishap.

All of the animals which were subinoculated with infected squirrel blood proved refractory to the infection. These failures may have been due to the relatively small numbers of asexual forms of the parasite present in the donor squirrels when the experiments performed.

Identification of the Plasmodium.

It seems almost certain that the plasmodium described above is the same as that observed in Malabar squirrels from the same locality by Donovan (loc.cit). He did, however, record that it was "very like P.vivax of man". The parasite described above can readily be distinguished from P.vivax of man by the absence of enlargement and pallor of the infected erythrocytes and by the absence of strippling similar to Schuffners dots.

Donovan (loc.cit.) proposed the name P.ratufae sps.nov. for the parasite he observed, although he states that "Vassal found a similar, if not identical, parasite in a squirrel (Sciurus griseimanus) in Annam".

A careful study of the description of the squirrel parasite given by Vassal (1905a, 1905b) and for which Laveran (1905) proposed the name P.vassali, failed to reveal any criterion by which it could be differentiated from the plasmodium described above. In a later paper, Vassal (1907)

reported the occurrence of P.vassali in other species of squirrel in Annam including Sciurus vittatus, Sciurus vulgaris and another unidentified species. In view of the close morphological similarity between the plasmodium of the Malabar squirrel and P.vassali it is not justifiable to give a new specific name to the former as was suggested by Donovan. A convenient taxonomic expedient can be used in classifying the plasmodium of the Malabar squirrel as P.vassali var. ratufae. If further work proves that this parasite is a distinct species it can be given specific rank as P.ratufae, or conversely, should this parasite prove to be identical with P.vassali the varietal name can be omitted. It is certainly not justifiable to assume that P.vassali and P.vassali var. ratufae are distinct species solely on the grounds that they have been recorded from different countries and different species of squirrel.

SUMMARY.

A description has been given of a plasmodium of the Malabar squirrel (Sciurus indicus malabaricus). It is considered that this parasite is identical with that described by Donovan in Malabar squirrels shot in the same area. Donvan's taxonomic classification as P.ratufae sps. nov. has been criticised and reasons advanced for naming the parasite P.vassali var. ratufae. Unsuccessful attempts to transmit the plasmodium to common laboratory animals were made.

ENVOI.

As I walked down the footpath from the Institute to Kasauli for the last time on August 12th, 1947, I recalled how Christophers, Sinton, Shortt, Covell and Mulligan had trod the selfsame path. I thought also of Ross and James who in other parts of India had furthered our knowledge of malaria. The mantle had passed from generation to generation and I was the last in the line of officers of the Indian Medical Service to serve at Kasauli. We had all made our contributions great and small in India but the malaria problem was still immense. "Science moves but slowly, slowly, moving on from point to point".

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